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(71) Applicants *(for all designated States except US)*: AMGEN INC. [US/US]; 1840 Dehavilland Drive, Thousands Oaks, CA 91320-1789 (US). UNIVERSITY OF SOUTHERN CALIFORNIA [US/US]; 3716 S. Hope Street, #313, Los Angeles, CA 90007-4344 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)* : BURNETTE, W., Neal [US/US]; 1724 Campbell Avenue, Thousand Oaks, CA 91360 (US). KASLOW, Harvey, R. [US/US]; 2430 Kenilworth, Los Angeles, CA 90039 (US).

(74) Agents: ODRE, Steven, M. et al.; Amgen Inc., 1840 Dehavilland Drive, Thousand Oaks, CA 91320-1789 (US).

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(54) Title: RECOMBINANT DNA-DERIVED CHOLERA TOXIN SUBUNIT ANALOGS

(57) Abstract

The development of subunits and subunit analogs of the cholera exotoxin by recombinant DNA techniques provides vaccine products that can retain their biological activity and immunogenicity, and can confer protection against disease challenge. Genetically-enginneered modifications of the subunits result in products that retain immunogenicity, yet are reduced in, r are essentially free of, enzymatic activity associated with toxin reactogenicity.

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RECOMBINANT DNA-DERIVED CHOLERA TOXIN SUBUNIT ANALOGS

BACKGROUND OF THE INVENTION

Field Of The Invention

The present invention relates to the recombinant expression of analog subunits of cholera exotoxin, and to vaccines based on such analogs. More particularly, genetically engineered modifications of the exotoxin provide analogs of cholera toxin having the capability to elicit a protective response with reduced or essentially no catalytic activity which can contribute to the reactogenicity of cholera vaccines.

Description Of The Art

The term "cholera" refers to the disease caused by infection with the etiologic agent *Vibrio cholerae*, most commonly occurring in geographical areas where poor hygienic conditions prevail. Cholera remains a major cause of morbidity and mortality in many parts of the world(1,2). Experience has shown that contraction of the disease usually confers long-lasting protection against subsequent exposure to the etiologic agent(3). Consequently, considerable effort has been devoted to the development of a vaccine that would be similarly protective. A parenteral whole cell cholera vaccine has been produced, but some no longer regard it as useful, particularly for young children who are at greatest risk from the disease(1).

As for many other infectious diseases, a biological exotoxin (in this case, "cholera toxin" or "CTX") encod d by the g nome of th inf ctious agent and secreted by it, contribut s significantly to the ability of the microorganism to colonize the infected

host(4). Mor ov r, exposure to th toxin causes sev r diarrh a and vomiting which result in dehydration, a lif -thr at ning condition of th dis as (3,5). Th se experiences suggest that a vaccine which elicits an immunologic response (e.g., antibodies) sufficient to neutralize the toxin would thus significantly help to prevent or reduce bacterial colonization and attendant symptoms such as diarrhea and vomiting. Thus, substantial effort has been applied toward developing a vaccine containing a non-toxic analog of the toxin, i.e., a "toxoid"(1,3-13). It is known that cholera toxin is a multi-subunit macromolecule consisting of a subunit termed "A", containing a catalytic region called "A1" which ADP-ribosylates G-proteins in target cells, and a "B" oligomer which binds the holotoxin to the target cells(6). Non-toxic analogs of cholera toxin have been produced for purposes of vaccine development by various means. These methods include chemical treatment of the holotoxin or toxin subunits, deletion of the A subunit and use of the remaining B oligomer, and synthesis or isolation of peptide fragments of toxin subunits(1,3-13).

In recent years, efforts have turned toward the development of oral vaccines, with two approaches apparently having received the most attention. One of these approaches is based on the use of killed *V. cholerae* (i.e., chemically- or heat-inactivated), alone, or supplemented with the B oligomer of cholera toxin(1,11,12). This approach has been found to produce incomplete protection, particularly in young children(12). The other approach involves the use of living, but attenuated, strains of *V. cholerae* which fail to produce the A1 subunit of the toxin(13). Vaccin s of this kind have provid d gr at r levels of protection, but until recently hav also be n associat d with unacceptable intestinal side-effects.

A recently-developed vaccine based on *V. cholerae* strain CVD 103-HgR, in which the gene encoding the A subunit is omitted, appears to be better tolerated, at least in adults(13). However, to our knowledge, this vaccine has not been tested in children or in large-scale clinical trials.

Recent studies on the nature of cholera toxin have provided insights concerning its structure that may have application in vaccine development based on a recombinant approach. It is known that naturally-ocurring subunit A is synthesized in *V. cholerae* as a preprotein(14), which is subsequently cleaved to proteolytically remove a signal peptide sequence of approximately 2,160 kDa. Further post-translational processing yields an amino-terminal polypeptide of approximately 21,817 kDa (subunit A1) and a carboxyl-terminal polypeptide of approximately 5,398 kDa (subunit A2), which are linked by a disulfide bridge(6,15,16); reduction of the disulfide bond is believed necessary for catalysis of the ADP-ribosyltransferase reaction (6,15,16). Likewise, the B subunit is synthesized as a preprotein which is subsequently cleaved by protease to remove a signal peptide. The genes, or cistronic elements, for the A1, A2 and B subunits of cholera toxin have all been fully sequenced and described in the literature(16).

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1A is the DNA sequence of the cistronic element encoding the A subunit of CTX from the prior art. The single-letter amino acid sequence beneath the DNA sequence indicates the proposed open reading frame for the A polypeptide. Subregions are also indicated, showing the start of the signal peptide (pre-A), A1, two proposed sites for carboxyl-terminal

processing of A1, and the proposed start and termination of A2. It should be noted that the literature provides inconclusive evidence as to the exact location of the carboxyl terminus of A1(16,17).

FIGURE 1B is the DNA sequence of the cistronic element encoding the B subunit of CTX. Initiation and termination codons and proposed cleavage sites are likewise shown. Interestingly, the region of DNA in the operon encoding the termination of A2 and the initiation of B overlap; these two proteins, however, are in different reading frames.

FIGURE 2 shows schematic structures for the preprotein and processed protein forms of the A and B subunits of native CTX and the forms of the recombinant subunits. The "squiggle" at the amino termini of the preprotein species represents the signal peptide which is removed by *V. cholerae*. "M" indicates an amino terminal methionine residue; "(M)" indicates that this is a heterologous (non-native) residue residing at the amino terminus of the mature recombinant CTXA and CTXA1 subunits, and analogs thereof; amino acid sequence data indicates that the heterologous methionine residue is not substantially cleaved from the recombinant polypeptide by cellular methionine amino-peptidase. "S" indicates the sulfur moiety involved in a disulfide linkage between cysteine residues. Other selected amino acids are indicated by their standard single-letter codes, with their position within the polypeptides indicated. Selected restriction enzyme cleavage sites for the encoding DNA sequences are indicated on the encoded polypeptide with their standard three-letter codes. Native ("n") CTXA is believed to be synthesized in *V. cholerae* as a preprotein ("pre-A"), containing an amino-terminal

signal sequence. Post-translational processing results in cleavage of the signal to yield mature CTXA. Perhaps simultaneously, a small portion of the carboxyl terminus is also cleaved proteolytically. The larger A fragment (CTXA1) and the smaller carboxyl-terminal A fragment (CTXA2) are held together after cleavage by a disulfide bridge between the single cysteine residue in each fragment. The literature possesses conflicting reports as to the location of the terminus of CTXA1 (either Arg^{192} or Ser^{194}); CTXA2 is believed to begin with Met^{195}. Native ("n") CTXB is also synthesized with an amino-terminal signal sequence that is subsequently processed by protease. Interestingly, the region of the CTXB cistronic element encoding its amino terminus overlaps with the CTXA cistronic element encoding its carboxyl terminus; the coding sequences, however, are in different reading frames(16). Recombinant ("r") CTXA was synthesized in *E. coli* under control of an optimized expression vector. An oligonucleotide linker (NdeI-XbaI) was used for cloning of the left-hand end of the DNA element, substituting an initiating methionine codon for the signal peptide-encoded sequence. The A2 region was not removed from A1 in the recombinant *E. coli*. A similar left-hand cloning strategy was used for CTXB, except an NdeI-AccI fragment was used to substitute the methionine initiation codon for its signal peptide-encoded sequence. Recombinant CTXA1 was synthesized to mimic native, reduced CTXA1. In this regard, an oligonucleotide linker at the right-hand end was used to substitute a termination codon for the A2 sequence such that A1 terminates at Ser^{194}, one of the two proposed cleavage sites in native CTXA1. Termination at Arg^{192} can also be easily accomplished using the same linker strategy. As previously noted, the amino terminal methionines of the recombinant CTXA and CTXA1

molecul s, and their analogs, are not beli ved to be substantially removed by nascent *E. coli* methionine aminopeptidase.

FIGURE 3 is the SDS-PAGE of native and recombinant CTX subunits. Recombinant CTXA, CTXA1, the $Arg^7 \rightarrow Lys$ analogs of recombinant CTXA and CTXA1, and recombinant CTXB were synthesized in *E. coli* and inclusion bodies prepared as described in the text. The inclusion body preparations, as well as purified commercial-grade native CTX, CTXA, and CTXB, were solubilized and subjected to SDS-PAGE under reducing conditions. Lane 1, native CTX; lane 2, rCTXA/A7; lane 3, rCTXA $Arg^7 \rightarrow Lys$ analog (rCTXA/L7); lane 4, rCTXA1/A7; lane 5, rCTXA1 $Arg^7 \rightarrow Lys$ analog (rCTXA1/L7); lane 6, rCTXB; lane 7, native CTXB; lane 8, native CTXA (only CTXA1 is visualized). Subsequent to electrophoresis, the gel was stained with Coomassie Brilliant Blue R250 and then destained to reveal the stain-retaining polypeptides.

FIGURE 4 is the SDS-PAGE and autoradiographic analysis of rCTXA1 and CTXA1 analog ADP-ribosyltransferase activity. In Panel A, native CTXA, recombinant CTXA1, and various site-specific analogs or preparations of rCTXA1 were subjected to SDS-PAGE and stained with Coomassie Blue. These same preparations were used as enzyme sources to ADP-ribosylate membrane-associated G protein using [^{32}P]NAD under assay conditions described in the text. After the reactions were quenched, the entire reaction mixture from each preparation was subjected to SDS-PAGE, and the gel dried and subjected to autoradiography to visualize prot ins that have b en covalently modified by addition of [^{32}P]-labeled ADP-ribos . Panel B shows the result of the assays when no G-protein substrate was added,

illustrating the ability of recombinant CTXA1 to autoribosylat ; int r stingly, analog CTXA1/L7 has lost this r activity. Pan 1 C shows the ADP-ribosylation of substrate G protein found in human erythrocyte membranes. Addition of this substrate substantially shifts reactivity of the enzyme from itself (autoribosylation) to the target G protein (seen in the autoradiogram as its ribosylated α-subunit). Again, rCTXA1 analog L7 lacks this reactivity.

FIGURE 5 is the SDS-PAGE and autoradiographic analysis of rCTXA and rCTXA analog ADP-ribosyltransferase activities, similar to that shown for rCTXA1 in Figure 4. Because the rCTXA preparation possesses significantly lower activity than rCTXA1 (see Figure 6), presumably because the former still contains the uncleaved A2 "tail" at its carboxyl terminus, these autoradiograms were attained by a longer exposure of the gel (Panel A) to the x-ray film. Panel A is the stained SDS-polyacrylamide gel of the rCTXA proteins; in comparison with Figure 4, Panel A, it is evident that the recombinant expression of these proteins is generally less than that of the companion rCTXA1 proteins. The recombinant CTXA preparation was capable of autoribosylation (Panel B) and of ADP-ribosylating the G protein substrate in human erythrocyte membranes (Panel C); these activities are substantially diminished in comparison with rCTXA1. Nevertheless, the CTXA preparations exhibit the same general pattern of inactivation as do their CTXA1 counterparts. Again, the L7 analog (Arg7→Lys) is devoid of ADP-ribosylating activity.

FIGURE 6 is the SDS-PAGE and autoradiographic comparison of the ADP-ribosyltransferase activity of rCTXA and rCTXA1/L7 with that of rCTXA1 and rCTXA1/L7.

Panel A is the r activity without add d substrate and Pan l B is with human erythrocyt membran s added as substrate. Th lan s contain: lane 1) blank (no sampl added to reaction); lane 2) native CTXA without urea treatment; lane 3) native CTXA with urea treatment; lane 4) rCTXA; lane 5) rCTXA/L7; lane 6) rCTXA/L7 plus native CTXA; lane 7) rCTXA1; lane 8) rCTXA/L7; lane 9) rCTXA1/L7 plus native CTXA. This experiment demonstrates that the rCTXA preparation is much less active than rCTXA1 for ADP-ribosylation of G proteins (compare lanes 4 and 7), yet exhibits substantial autoribosylating activity. Confirming the data shown in Figures 4 and 5, substitution of lysine for arginine-7 in rCTXA and rCTXA1 abolishes their ribosylating activities, both for autocatalysis and for G protein. Retention of activity by native CTXA when added to the analog preparations (lanes 6 and 9) additionally illustrates that it is not a contaminant of the recombinant preparations that suppress this activity.

FIGURE 7 illustrates the ADP-ribosylation of H27 fibroblast and erythrocyte membranes by CTXA and CTXA1 analogs. Naturally-occurring CTXA or recombinant CTXA1 analogs were incubated with [^{32}P]NAD and either human erythrocyte or H27 fibroblast membranes. After incubation, the mixtures were precipitated, centrifuged, and the resulting pellets subjected to SDS-PAGE. The gels were stained with Coomassie Blue, dried, and subsequently exposed to x-ray film to produce autoradiograms. B, no CTXA or CTXA1 analog added; A, naturally-occurring CTXA, A+u, naturally-occurring CTXA treated with urea; rA1, r combinant CTXA1 with no residue substitutions; RBC, human rythrocyte membranes.

FIGURE 8 illustrates th ADP-ribosylation of H27 fibroblast and membran s by CTXA and CTXA1 analogs. Naturally-occurring CTXA or r combinant CTXA1 analogs were incubated with [^{32}P]NAD in the presence of èither human erythrocyte membranes, H27 fibroblast membranes, or no added substrate-containing membranes. After incubation, the mixtures were precipitated, centrifuged, and the resulting pellets were subjected to SDS-PAGE. The gels were stained with Coomassie blue, washed and dried. The upper left panel is a photograph of a stained gel of samples incubated in the absence of substrate-containing membranes; the upper right panel is an autoradiogram of this gel. The lower left and right panels are autoradiograms of gels of samples incubated with erythrocyte and H27 membranes, respectively. B, no CTXA or CTXA1 analog added; A, naturally-occurring CTXA; A + u, naturally-occurring CTXA treated with urea; rA1, recombinant CTXA1 with no residue substitutions; RBC, human erythrocyte membranes.

SUMMARY OF THE INVENTION

The present invention provides a recombinant DNA molecule, at least a portion of which encodes an analog of the catalytic subunit of cholera toxin having reduced enzymatic activity, such activity generally accepted to be associated with vaccine reactogenicity. More specifically, site specific mutagenesis, as described herein, results in analogs of the A and A1 subunits which, compared to the native toxin counterparts, exhibit a significant reduction in catalytic function as m asured by ADP-ribosyltransferase activity.

The term "catalytic subunit of cholera toxin" used in this disclosure r fers to both the

A region of cholera toxin and the A1 subr gion, as depicted in Figs. 1A and 2. Th s r gions of the chol ra toxin macromol cul ar known to possess ADP-ribosyltransferase catalytic activity(6). This enzyme is a complex of two sub-activities: an NAD glycohydrolase activity which cleaves NAD into nicotinamide and ADP-ribose, and a transferase activity which transfers the ADP-ribose to the G protein substrate. Measurements of the ADP-ribosyltransferase activity in this disclosure represent a summation of both activities. The present invention comprehends mutagenesized versions of these A and A1 polypeptides, and analogs or derivatives of such polypeptides, which in their native forms are sources of catalytic activity within the cholera toxin multimer.

The genetically-engineered analogs of cholera toxin, which are a product of this invention, provide recombinant DNA-derived materials suitable for use in vaccines for the prevention of cholera disease. The A and A1 subunit analogs can be used alone or in combination with B oligomer in a toxoid-based vaccine, or phenotypically expressed by variants of *V. cholerae*, or phenotypically expressed under the genetic control of other immunizing vectors. It should be noted that the analog A and A1 subunits of this invention are utilizable by themselves as antigenic agents in a vaccine because they may contain important protective epitopes. However, the use of these analogs in association with B subunits may be more desirable. The B oligomer contains neutralizing epitopes useful for eliciting immunoprotection(1,3,5). Association of the A subunit with the B oligomer may lead to a more effective immunogenic response against the B oligomer. Th B oligomer can be purified from *V. cholerae* or, alternatively, can be derived recombinantly in a manner similar to th A and A1 subunits by expression in

E. coli or other recombinant hosts, including other bact rial organisms (.g., *Salmonella typhimurium* or *typhi*, *Bacillus sp.*), yeast (e.g., *S. c r visiae)*, and viruses (e.g., vaccinia and adenoviruses).

Mutagenesis in accordance with this description enables production of mutants varying in diminished catalytic activity, ranging from variants which exhibit attenuated activity to those which are essentially free of such activity (i.e., less than 5%). This flexibility in approach is desirable because attenuation, rather than elimination, of catalytic activity may be helpful in providing a greater degree of and/or longer-lasting, protective response. Moreover, because of their diminished enzymatic activity, the analog subunits provided by this invention are expected to be less reactogenic.

DETAILED DESCRIPTION

The present invention provides high-level, direct recombinant expression of all CTX subunits necessary for vaccine production. Further, catalytic subunit analogs provide biological activity that is reduced in, or essentially free of, ADP-ribosyltransferase catalytic activity. The present analogs used alone, or in combination with B oligomer of the toxin (whether derived from natural sources or by recombinant means), can provide products that are useful in a vaccine and greatly reduce the likelihood of side-effects generally accepted to be associated with the catalytic activity in the native toxin. The toxin analogs of the present invention can be formulated into vaccine compositions or us d in combination with oth r immunogenic agents in a multi-component vaccine.

Th individual cistronic lements, or portions th r of, encoding th A and B subunits of *V. chol rae* toxin were subclon d and dir ctly expr ssed individually in a recombinant host cell system (i.e., *E. coli*). In the absence of a native signal peptide (substituted with a methionine to initiate translation), high levels of expression, in the range of 2% to 80% of total cell protein, were obtained. The fermentation of expressor cells resulted in mature species of rCTXA, rCTXA1 and rCTXB, as shown in Fig. 3. It should be noted that rCTXA is not processed to rCTXA1 and rCTXA2 in *E. coli*, presumably due to the absence of the specific enzyme or a failure of rCTXA to be compartmentalized with this enzyme. Thus, rCTXA possesses the A1 sequence covalently linked to the A2 sequence.

Amino acid analysis of selected recombinant molecules demonstrated that the heterologous (non-native) methionyl residue is not substantially removed from the various rCTX and rCTXA1 subunit species by cellular methionine aminopeptidase; thus, these are also methionyl-mature analogs. All of the recombinant proteins were recovered as inclusion bodies from lysed cells. The subunits were found to have migration patterns in reducing SDS-PAGE essentially identical to authentic native subunits, with the exception of rCTXA which is not processed in *E. coli* to result in cleavage of the A2 region from A1. As shown in Fig. 3, high-level recombinant expression of subunits CTXA, CTXA1 and CTXB in *E. coli* was achieved by direct, non-fusion means.

Although alternativ methods and materials can be us d in the practice of the pr sent invention, the pref rr d methods and materials ar d scribed

below. All references cited hereunder are incorporated herein by reference.

MATERIALS AND METHODS FOR RECOMBINANT EXPRESSION OF CTXA, CTXA1 AND CTXB SUBUNITS

Materials. DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA), Bethesda Research Laboratories (Gaithersburg, MD), Boehringer Mannheim Biochemicals, (Indianapolis, IN), and International Biotechnologies, Inc. (New Haven, CT); enzymes were used according to manufacturer recommendations. All chemicals and biochemicals were analytical reagent grade. Purified, naturally-occurring cholera toxin and toxin subunits were purchased from Sigma Chemical Company (St. Louis, MO) and List Biologicals (Campbell, CA). Synthetic oligonucleotides were synthesized based on methods developed from the chemical procedure of Matteucci and Caruthers(18).

Plasmids and Bacterial Strains. Plasmids pRIT10810 and pRIT10841, (ATCC 39051 and ATCC 39053, respectively), containing the portions of the CTX operon, were obtained from the American Type Culture Collection, Rockville, MD. Expression plasmids pCFM1036, pCFM1146 and pCFM1156 were derived at Amgen.

A description of the expression vector system used herein is described in United States Patent No. 4,710,473 (Morris), which is incorporated herein by reference. Such plasmids contain an inducible promoter, a synthetic ribosome binding site, a cloning cluster, plasmid origin of replication, a transcription terminator, genes for regulating plasmid copy number, and a Kanamycin resistance gene. The derived plasmids differ from each other in a number of respects. The plasmid

pCFM1036 can be derived from pCFM836 (see U.S. 4,710,473) by substituting the DNA sequence between the unique AstII and EcoRI restriction sites containing the synthetic P_L promoter with the following oligonucleotide:

AatII EcoRI

5′-CATCGATTCTAG-3′
3′-TGCAGTAGCTAAGATCTTAA-5′

This plasmid contains no inducible promoter preceding the restriction cluster. The plasmid pCFM1146 can be derived from pCFM836 by substituting the small DNA sequence between the unique ClaI and XbaI restriction sites with the following oligonucleotide:

ClaI XbaI

5′-CGATTTGATT-3′
3′-TAAACTAAGATC-5′

and by destroying the two endogenous NdeI restriction sites by end-filling with T4 polymerase enzyme followed by blunt-end ligation. The plasmid contains no synthetic ribosome binding site immediately preceding the restriction cluster. The plasmid pCFM1156 can be derived from pCFM1146 by substitution of the small DNA sequence between the unique XbaI and KpnI restriction sites with the following oligonuceotide which installs an optimized synthetic ribosome binding site:

XbaI KpnI

5′-CTAGAAGGAAGGAATAACATATGGTTAACGCGTTGGAATTCGGTAC-3′
3′-TTCCTTCCTTATTGTATACCAATTGCGCAACCTTAAGC-5′

Plasmids pBR322, pUC18, pUC19, and phage M13mp18 and M13mp19 DNA were purchas d from Beth sda Research Laboratories. *E. coli* FM5 cells were derived at Amgen Inc., Thousand Oaks, CA from *E. coli* K-12 strain(19) from C.F. Morris and contain the integrated lambda phage repressor gene, CI_{857}(20). Construction of the individual subunit expression plasmids is described herein. Vector production, cell transformation, and colony selection were performed by standard methods(21).

Analytical Procedures. DNA sequencing was done by modification of the primer-extension, chain-termination method(22,23). Protein sequence analyses were performed by automated Edman degradation in an ABI 470A gas-phase microsequenator(24,25) and by standard enzymatic means, the latter to obtain carboxyl-terminal sequences of selected-proteins. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli(26), and elution of polypeptides from polyacrylamide gels was similar to the method of Hunkapiller *et al.*(27). The ratio of recombinant protein to total cellular protein or total inclusion body protein was assessed by SDS-PAGE of whole-cell lysates followed by staining with Coomassie Brilliant Blue R250 and subsequent gel scanning by integrative densitometry.

Assays for the measurement of ADP-ribosyltransferase catalytic activity were done as follows: Native CTXA and recombinant subunits were incubated in a solubilization buffer of 8 M urea, 25 mM sodium phosphate (pH 7.0) and 10 mM dithiothreitol (DTT) for one hour at 37°C and centrifuged at 10,000 rpm for 15 minut s without refrigeration. The additions to the solubilization buffer were adjust d to yield 1 µg of native or recombinant A1 per 4 µL, which

was then added to 60 µL of a reaction mixture (see below) and incubated for one hour on ice.

Reaction Mixture

Reagent*:	(final)/60 µl	(final)/100 µl
Na_xPO_4, pH 7.0, 1 M	416 mM	250 mM
DTT, 100 mM	5 mM	3 mM
GTP, 10 mM	167 µM	100 µM
Thymidine, 100 mM	17 mM	10 mM
$MgCl_2$, 1 M	5 mM	3 mM
[32P]-NAD	2.5 µCi	2.5 µCi
NAD, 2500 µM	50 µM	30 µM

*The reagents were obtained from commercial sources. Naturally-occurring CTXA was acquired from List Laboratories. As a control, native CTXA was also assayed by incubation in the same buffer as above, but without urea, for 15 minutes at 37°C, then kept on ice until assayed for ADP-ribosyltransferase activity.

Thirty-six µL of water or a buffer containing human erythrocyte membranes(28) were added to yield a final volume of 100 µL for each sample and the samples incubated at 30°C. After 30 minutes, the reaction was terminated by adding 50 µL of 5 mM NAD and 0.03% sodium deoxycholate to each sample and the reaction mixture chilled on ice for 10 minutes. Fifty µL of 40% trichloroacetic acid (TCA) were then added, the samples placed on ice for at least 15 minutes; 2 mL of water were subsequently added to each sample, and the precipitated protein pelleted by centrifugation. The supernatants were removed and the pelleted protein was frozen. On the following day, the pelleted protein was

subject d to SDS-PAGE(26,29). The g l was stained with Coomassi Brilliant Blue, destain d, dri d and subj cted to autoradiography to measur th cont nt of covalently linked [^{32}P]-labeled ADP-ribose in the proteins of the various bands. An approximation of the specific activities of the recombinant CTXA1 and recombinant analog CTXA1 proteins (relative to the activity of native CTXA1) was obtained by densitometric scanning of the gels and autoradiograms. The stained gels were scanned to approximate the amount of individual protein added to each reaction mixture. The autoradiograms were scanned to estimate the amount of [^{32}P]ADP-ribose transferred to the G protein substrate as a function of the density of the autoradiographic image.

Construction of Expression Plasmids. All expression plasmids were constructed from a series of *E. coli* generalized expression vectors differing as described previously. The individual cholera toxin subunit gene segments were isolated using the restriction sites shown in Figs. 1 and 2. The upstream restriction site was just inside the codon for the amino-terminal residue of the mature, processed form of the subunit (i.e., without the signal sequence). For purposes of recombinant expression in *E. coli*, the portion of the CTX genes encoding their native signal peptides were deleted and substituted instead by a methionine initiation codon, for expression of the "methionyl-mature" form of the subunit analogs. Synthetic oligonucleotide linkers were employed to effect insertion of the gene segments into the expression plasmids at an optimal distance downstream of th synth tic promoter and ribosome binding sit . The upstream linkers restor d the reading frame of each gene back to the first codon of

th matur amino t rminus; th oligonucleotides included a methionyl initiation codon.

Following transformation of *E. coli* FM5 cells with the various plasmid constructs and plating on Kanamycin-containing agar, appropriate numbers of colonies were selected, replica-plated, grown as small liquid cultures ("minipreps"), and induced at 42°C for 4 hours. The minipreps were then screened by light microscopy for the presence of inclusion bodies in the bacterial cells. Preparations exhibiting apparent inclusions were identified and matching colonies from the replica plates subjected to flask-scale laboratory fermentation at the induction temperature. Samples were removed from fermentation at various times post-induction and examined for the appearance of the appropriate CTX subunit by SDS-PAGE followed by Coomassie Brilliant Blue-staining. The structure of the plasmid from each expression clone was confirmed by restriction mapping of the isolated plasmid and verified by DNA sequencing of junction regions.

Expression of Recombinant CTX, CTXA1 and CTXB. When *E. coli* cells containing, separately, the CTXA expression plasmid (pCTXA/A7/1156), the CTXA1 expression plasmid (pCTXA1/A7/1156), and the pCTXB expression plasmid (pCTXB/1156) were fermented at 37°C and 42°C, they produced major intracellular proteins (Figure 3) of approximately 27,215 daltons, 21,817 daltons and 11,600 daltons, respectively; recombinant CTXA1 and CTXB comigrated with authentic (native) CTXA1 and CTXB, respectively, in SDS-PAGE. Our recombinant CTXA has no native counterpart, since natural CTXA is cleaved to CTXA1 and CTXA2 by *V. cholerae* protease at some point before secretion from the organism; A1 and A2 ar held tog ther by a disulfide bond that is r duc d by the buffers used in SDS-PAGE. Partial amino acid s quence analysis stablished that r combinant

polypeptide CTXA1/A7 and CTXA1/L7 (see description below) had the amino terminal sequence predicted for the native CTXA1 subunit, but that the heterologous initiating methionine residue is not substantially removed.

Properties of Recombinant CTX Subunits.

Very little, if any, of the CTX subunits appear to be secreted from the *E. coli* cells. The bulk of each subunit was found in the form of inclusion bodies and constituted 2% to 80% of total cellular protein. Cell lysis by French press and low speed centrifugation resulted in pellet fractions that contained up to 80% of their protein as the individual subunits. All the rCTX subunits were detectable in gels stained with Coomassie Brilliant Blue (Figure 3).

CTXA AND CTXA1 ANALOGS

Using techniques of protein engineering and site-specific mutagenesis(19,30), CTXA and CTXA1 analogs were made. From those analogs made and tested by the time of this submission, it was found that mutagenesis of the amino acid residues at positions arginine-7, histidine-44, histidine-70, glutamic acid-112, and aspartic acid-9, and truncation of the carboxyl terminus (at tryptophan-179 of the mature native CTXA sequence) resulted in diminished or essentially no ADP-ribosyltransferase activity.

Construction of the CTXA Expression Plasmid.

Plasmid pRIT10841 (ATCC 39053) was cleaved with restriction enzymes XbaI and ClaI and a 552-bp DNA fragment was isolated by gel electrophoresis which contained the left-hand end of the CTXA gene to the region encoding the protease-sensitive portion that results in CTXA cleavage to CTXA1 and CTXA2. Plasmid pRIT10810 (ATCC 39051) was cleaved with restriction

enzyme ClaI and HindII (the latter an isoschizomer of HincII) and a 368-bp DNA fragment was isolated that encoded a portion of the CTXA subunit from the protease-sensitive site (encoded at the ClaI site) (16,17) through the CTXA2 region, past the termination codon of CTXA, and into the alternative open reading frame of the CTXB subunit.

A synthetic oligonucleotide linker was prepared to reconstitute the open reading frame of CTXA from the site encoding the first amino acid of the mature protein sequence (asparagine) to the XbaI site. This linker possessed NdeI cohesiveness at its left-hand end in order to generate a methionine initiation codon that would substitute for the sequence encoding the signal peptide and to facilitate insertion of the gene construction into the expression vector; the right-hand end of the linker possessed an XbaI overlap. This linker possessed the sequence:

5'-TATGAATGATGATAAGTTATATCGGGCAGATT-3'
3'-ACTTACTACTATTCAATATAGCCCGTCTAAGATC-5'

Plasmid pUC19 was digested with NdeI and XbaI and the linker above inserted. After ligation and transformation, a pUC plasmid named p2A/pUC19 was isolated that contained the linker sequence in place of the normal pUC19 NdeI-XbaI sequence.

Plasmid p2A/pUC19 was digested with XbaI and HincII. The large fragment from this digestion was ligated together with the 552-bp XbaI-ClaI DNA fragment containing the left-hand end of the CTXA gene and the 368-bp ClaI-HindII DNA fragment containing the right-hand end of the CTXA gene (past the termination codon and into the alternative open reading frame of the CTXB subunit). This produced a new plasmid containing th

entire matur CTXA gene; this plasmid was called pCTXA/A7/pUC19.

The *E. coli* xpression plasmid pCFM1156 was digested with NdeI and HindIII to remove this small portion of its cloning cluster. Plasmid pCTXA/A7/pUC19 was also digested with NdeI and HindIII, and a DNA fragment (772-bp) containing the entire region of the CTXA gene was isolated. This fragment was subsequently ligated into the digested pCFM1156 plasmid to produce the CTXA expression plasmid pCTXA/A7/1156. This NdeI-NdeI fragment could be inserted into pCFM1156 in either of two orientations, only one of which would produce an open reading frame giving rise to a large protein when expressed. This clone was selected (by analysis of induced clones by SDS-PAGE to identify the recombinant CTXA protein) and the proper orientation confirmed by DNA sequencing at the upstream NdeI junction region.

Construction of the CTXB Expression Plasmid.

Plasmid pRIT10810 (ATCC 39051) was digested with ClaI and BstXI and a 538-bp DNA fragment was isolated; this contained the the A2 coding region of CTXA, the entire CTXB coding region, and a short DNA sequence to the right of the termination codon of CTXB.

A synthetic oligonucleotide linker was prepared that permitted the cloning of the right-hand end of the DNA sequence above into pUC19. This linker possessed BstX1 and HindIII cohesive ends and had the sequence:

5'-GTGGAATTCGGTACCATGGA-3'
3'-GAGTCACCTTAAGCCATGGTACCTTCGAA-5'

Plasmid pUC19 was digested with HindIII and AccI (the latt r generating a coh siv end compatible with that generated by ClaI). The larg pUC19 fragment

was ligat d with th 538-bp ClaI-BstXI DNA fragment containing th CTXB and th BstXI-HindIII linker to produce a plasmid call d pCTXB/pUC19. This plasmid was then digested with HindIII and SspI (the latter just inside the initiation codon for CTXB and downstream from the ClaI site) to isolate a 345-bP SspI-HindIII fragment.

A synthetic oligonucleotide linker was prepared that possessed NdeI and SspI cohesive ends and the sequence:

5'-TATGACACCTCAAAAT-3'
3'-ACTGTGGAGTTTTA-5'

Plasmid pCFM1156 was digested with NdeI and HindIII to remove this portion of its cloning cluster. The large pCFM1156 DNA fragment was then ligated with the 345-bp SspI-HindIII fragment containing a portion of the CTXB gene and the NdeI-SspI linker that restored its left-hand coding region and insinuated a methionine codon at the left of this coding region to initiate protein synthesis. The subsequent expression plasmid, containing the entire CTXB gene with a methionine initiation codon, was called pCTXB/1156.

Linker Mutagenesis. An oligonucleotide linker called L7 was synthesized to substitute a lysine codon for that of arginine-7 in CTXA. The sequence of this linker, with NdeI and XbaI cohesive ends, is shown in Table 1. The L7 linker was cloned into the NdeI-XbaI site of pUC19 to produce a plasmid called pL7/pUC19. Plasmid pL7/pUC19 was then digested with XbaI and HindIII to remove this portion of the pUC19 cloning cluster and replaced through ligation with the 552-bp XbaI-ClaI DNA fragment containing the l ft-hand nd of the CTXA gen (see above) and th 368-bp ClaI-HindII DNA fragment containing the right-hand end of this g ne

(see above). This plasmid, called pCTXA/L7/pUC19, was digested with NdeI, and a 772-bp DNA fragment was isolated that possessed the entire mature CTXA gene with a substitution of the arginine-7 codon by a lysine codon. Plasmid pCFM1156 was digested with NdeI and ligated with the NdeI DNA fragment from pCTXA/L7/pUC19. This ligation produced a plasmid called pCTXA/L7/1156 - for expression of the mature form of an $Arg^7 \rightarrow Lys$ analog of CTXA in *E. coli*. As with the case of pCTXA/A7/1156 (above), it was necessary to select a clone containing this plasmid with the DNA insert in the proper open reading frame for synthesis of rCTXA/L7.

Oligonucleotide linkers 1E and 1F were synthesized to individually substitute, respectively, a phenylalanine codon for that of tyrosine-6 and a glutamate codon for that of aspartate-9. These linkers possessed NdeI and XbaI cohesive ends and had the sequences shown in Table 1. Plasmid pCTXA/A7/pUC19 (see above) was digested with XbaI and HindIII, and a 938-bp DNA fragment containing the right-hand portion of the CTXA gene was isolated. Plasmid pCFM1156 was digested with NdeI and HindIII to remove this short region of its cloning cluster. This segment was replaced by ligation with the NdeI-XbaI linker containing either the $Tyr^6 \rightarrow Phe$ or the $Asp^9 \rightarrow Glu$ codon mutation (linkers 1E and 1F, respectively) and the 938-bp DNA fragment of the CTXA gene. This produced two plasmids, pCTXA/1E/1156 and pCTXA/1F/1156, for expression of the mature forms of the CTXA analogs $Tyr^6 \rightarrow Phe$ and $Asp^9 \rightarrow Glu$, respectively, in *E. coli*.

The substitutions of sequences encoding mutations of glutamine for proline-185 and alanine for cysteine-187 resulted in CTXA gene fragments encoding only the CTXA1 portion of the CTXA subunit (see below for construction of the native-sequence CTXA1 gene and

th L7, 1E, and 1F substitution analogs of CTXA1 from the CTXA gen and its substitution analogs, r sp ctiv ly). Oligonucleotid linkers 1G and 1H w re synthesized to individually substitute, respectively, glutamine for proline-185 and alanine for cysteine-187. These linkers had DsaI and HindIII cohesive ends and possessed the sequences shown in Table 1. To effect the construction of the expression plasmids encoding the analog proteins, a 537-bp NdeI-DsaI DNA fragment was isolated from plasmid pCTXA/A7/pUC19. Plasmid pCFM1156 was then digested with NdeI and HindIII to remove this short segment of its cloning cluster. This segment was replaced by ligation with the 537-bp DNA fragment from pCTXA/A7/pUC19 and either 1G or 1H synthetic oligonucleotides. The linkers, in addition to encoding the specific amino acid substitutions, eliminate from the CTXA gene that portion encoding the A2 region of the CTXA subunit; thus, these mutations are exclusively in CTXA1 versions of the subunit. The resulting plasmids for expression of the $Pro^{185}\rightarrow Gln$ and $Cys^{187}\rightarrow Ala$ analogs of CTXA1 were called pCTXA1/1G/1156 and pCTXA1/1H/1156, respectively.

A plasmid expressing a carboxyl-terminal truncated version of CTXA1 terminating at Trp^{179} was constructed. This was accomplished by first digesting plasmid pCFM1156 with NdeI and HindIII to remove this short DNA fragment. Into this site in pCFM1156 was ligated the 537-bp NdeI-DsaI fragment from pCTXA/A7/PUC19 (see above) and a synthetic DNA fragment with DsaI and HindIII cohesive ends, and having the sequence:

5′-CGTGGTAATGATAGA-3′

3′-CATTACTATCTTCGA-5′

This plasmid, for expr ssion of CTXA1 truncated at Trp^{179}, was called pCTXA1/T1/1156.

Mutagenesis By Site-directed Priming.

Mutagenesis by site-directed priming was accomplished with kits of the "Altered Sites™ *in vitro* Mutagenesis System" purchased from Promega Corporation (Madison, WI); details of the experimental protocols for this procedure are contained in the technical manual available from Promega Corporation (printed 1/90).

To facilitate mutagenesis, a 938-bp XbaI-HindIII DNA fragment encoding a portion of the CTXA subunit was isolated from plasmid pCTXA/A7/pUC19 (see above). This fragment was cloned into the pSELECT1 phagemid vector from Promega. After packaging with helper phage, this vector contained a negative-sense copy of the CTXA fragment. A series of single-stranded, positive-sense DNA primers were synthesized to effect mutagenesis; the sequences of these primers (1B, 1C, 1D, and 1I) are shown in Table 1. These primers were individually annealed with the single-stranded phagemid containing the CTXA gene fragment; double-stranded phagemids were subsequently produced which contained the gene fragment and the individual codon substitutions encoded by the primers.

For preparation of plasmids capable of expressing the CTXA and CTXA1 subunit analogs containing a lysine substitution for arginine-146, a 207-bp BstXI-ClaI DNA fragment was isolated from the double-stranded phagemid containing the $Arg^{146}\rightarrow Lys$ codon mutation (1I). A 375-bp NdeI-BstXI DNA fragment and a 386-bp ClaI-HindIII fragment (for the CTXA version) containing a portion of the CTXA gene were isolated from plasmid pCTXA/A7/pUC19. Plasmid pCFM1156 was digested with NdeI and HindIII to remove this short portion of its cloning cluster. For construction of the CTXA v rsion of the $Arg^{146}\rightarrow Lys$ mutation, the

digested pCFM1156 plasmid was ligated with the 375-bp Nd I-BstXI fragment from pCTXA/A7/pUC19, the 209-bp BstXI-ClaI fragm nt from the double-strand d phagemid, and the 386-bp ClaI-HindIII DNA fragment from pCTXA/A7/pUC19. This resulted in a plasmid called pCTXA/1I/1156 for expression of the $Arg^{146}\rightarrow Lys$ analog of the CTXA subunit in *E. coli*. For construction of this mutation in the CTXA1 version of the subunit, the digested pCFM1156 plasmid was ligated with the 375-bp NdeI-BstXI fragment from pCTXA/A7/pUC19, the 209-bp BstXI-ClaI fragment isolated from the double-stranded phagemid, and a synthetic oligonucleotide linker that replaces a region of CTXA encoding the A2 portion of CTXA with a DNA sequence encoding the end of the A1 region and including a codon that terminates polypeptide synthesis at the end of CTXA1. This linker possessed ClaI and HindIII cohesive ends and had the sequence:

5′CGTAATAGGCGGCCGCA-3′
3′-ATTATCCGCCGGCGTCGA-5′

The resultant plasmid for expression of the $Arg^{146}\rightarrow Lys$ analog of CTXA1 in *E. coli* was called pCTXA1/1I/1156.

Preparation of plasmids capable of expressing individual analogs of CTXA containing the substitutions of $His^{44}\rightarrow Asn$, $His^{70}\rightarrow Asn$, or $Glu^{112}\rightarrow Gln$ was facilitated with primers (1B, 1C, and 1D, respectively) having the sequences shown in Table 1. After annealing of the primers individually to the pSELECT1 phagemid containing the 938-bp XbaI-HindIII CTXA fragment from pCTXA/A7/pUC19 (see above) and recov ring double-stranded plasmid, the r gions containing the site-specific mutations w re excis d from the plasmid by digesting with XbaI and HindIII,

and recovering a 938-bp DNA fragment in each case. Plasmid p2A/pUC19 (containing an Nd I-XbaI linker encoding the left-hand end of the mature CTXA; see above) was digested with XbaI and HindIII to remove this short region of the pUC19 cloning cluster to the right of the linker insert; this region was replaced by ligation with the 938-bp XbaI-HindIII fragment from the plasmid containing a single codon replacement. This series of pUC-derived plasmids were called pCTXA/1B/pUC19, pCTXA/1C/pUC19, and pCTXA/1D/pUC19, depending upon the codon replacement they contained. A DNA fragment containing the codon replacement was subsequently excised from each of these plasmids. Plasmid CTXA/A7/pUC19 was digested with BstXI and HindIII and a 593-bp DNA fragment was isolated. Plasmid pCFM1156 was digested with NdeI and HindIII to remove this short region of its cloning cluster, as described earlier, and this replaced by ligation with the individual CTXA analog gene inserts recovered from the pUC transition plasmids above and the 593-bp BstXI-HindIII DNA fragment from pCTX/A7/pUC19. When isolated, these new plasmids for expression of the site-specific analogs $His^{44}\rightarrow Asn$, $His^{70}\rightarrow Asn$, and $Glu^{112}\rightarrow Gln$ of CTXA in *E. coli* were called pCTXA/1B/1156, pCTXA/1C/1156, and pCTXA/1D/1156, respectively.

Conversion of CTXA and CTXA Analog Genes to CTXA1 and CTXA1 Analog Genes. With the exception of the plasmid containing the 1I codon substitution (pCTXA1/1I/1156), which was constructed during the mutagenesis process to lack the A2-encoding region, it was useful to convert the CTXA gene-containing and selected individual analog gene-containing expression plasmids to CTXA1 expression plasmids in order to express the A1 truncated version of CTXA that mimicked

the native species of this subunit in r duced holotoxin preparations. To p rform this conv rsion, it was n c ssary to del te a portion of th g n sequ nc of the CTXA gene (and the analog genes) to the right of the unique ClaI site. Although the actual site of polypeptide cleavage between the A1 and A2 regions has not been resolved in the prior art literature(16,17), it was decided to initially establish the carboxyl terminus of A1 at serine-194; it should be noted, however, that establishing the terminus at arginine-192 (the other terminus proposed in the literature) is a simple matter of inserting a new linker to substitute a termination codon immediately to the right of the arginine-192 codon.

For our purposes, each of the analog CTXA sequences (and the native CTXA sequence) we wished to convert to CTXA1 versions were excised from their pUC19 transition plasmids (*i.e.*, pCTXA/A7/pUC19, pCTXA/1B/pUC19, pCTXA/1C/pUC19, pCTXA/1D/pUC19, pCTXA/1E/pUC19, pCTXA/1F/pUC19, pCTXA/1G/pUC19, pCTXA/1H/pUC19) with restriction enzymes NdeI (at the sequence encoding the methionine initiation codon) and ClaI (at the site chosen for addition of a termination codon immediately to the right of the serine-194 codon); this DNA fragment was 585-bp in each case. For purposes of substituting a termination codon for the A2-encoding region and subsequent ligation of the gene segments into plasmid pCFM1156, an oligonucleotide linker was synthesized to possess ClaI and HindIII cohesive ends and had the following sequence:

5'-CGTAATAGGCGGCCGCA-3'
3'-ATTATCCGCCGGCGTTCGA-5'

Plasmid pCFM1156 was digested with NdeI and HindIII to remove this portion of its cloning clust r;

this region was replaced by ligation with the ClaI-HindIII link r and with an individual 585-bp DNA fragm nt from one of th pUC transition plasmids described above. Isolation of plasmid DNA following these ligations resulted in a series of plasmids capable of expressing CTXA1 and CTXA1 analog polypeptides in *E. coli*; plasmids prepared in this manner included pCTXA1/1B/1156, pCTXA1/1C/1156, pCTXA1/1D/1156, pCTXA1/1E/1156, and pCTXA1/1F/1156,.

Expression and Analysis of CTXA and Recombinant Analogs. Following preparation, each plasmid was used to transform a separate preparation of fresh, competent FM5 cells. Transformants were picked, grown as minipreps, induced to produce recombinant protein, and inclusion body-positive samples identified by light microscopy. These samples were fermented at a larger scale (≥ 1 liter) at the induction temperature to prepare greater amounts of each recombinant analog protein. Isolated cell pastes were lysed in a French press after resuspension in distilled H_2O with 1 mM DTT. Inclusion bodies were isolated from these lysates by simple low-speed centrifugation. These inclusion-body protein preparations contained as little as 2% and as much as 80% of the recombinant proteins. The samples were assessed for ADP-ribosyltransferase activity as previously described. The results obtained are shown in Figs. 4, 5, and 6 and in Table 2.

TABLE 1
CONSTRUCTION OF S1 ANALOGS

*	MUTATION	TECHNIQUE	OLIGONUCLEOTIDE SEQUENCE
L7	ARG7->Lys	Linker Insertion	5′-TATGAATGATGATAAGTTATATAAGGCAGATT-3′ 3′-ACTTACTACTATTCAATATATTCCGTCTAAGATC-5′
1B	His44->Asn	Site-directed Priming	5′-CCTTTATGATAACGCAAGAGGAA-3′
1C	His70->Asn	Site-directed Priming	5′GAGAAGTGCCAACTTAGTGGGTC-3′
1D	Glu112->Gln	Site-directed Priming	5′-AGATGAACAACAGGTTTCTGCTT-3′
1E	Tyr6->Phe	Linker Insertion	5′-TATGAATGATGATAAGTTATTCCGGGCAGATT-3′ 3′-ACTTACTACTATTCAATAAGGCCCGTCTAAGATC-5′
IF	Asp9->Glu	Linker Insertion	5′-TATGAATGATGATAAGTTATATCGGGCAGAAT-3′ 3′-ACTTACTACTATTCAATATAGCCCGTCTTAGATC-5′
1G	Pr 185->Gln	Linker Insertion	5′-CGTGGATTCATCATGCACCGCAGGGTTGTGGGAATGCTCCAAGATCATCGTAGA-3′ 3′-CTAAGTAGTACGTGGCGTCCCAACACCCTTACGAGGTTCTAGTAGCATCTTCGA-5′
1H	Cys187->Ala	Linker Insertion	5′-CGTGGATTCATCATGCACCGCCGGGTGCAGGGAATGCTCCAAGATCATCGTAGA-3′ 3′-CTAAGTAGTACGTGGCGGCCCACGTCCCTTACGAGGTTCTAGTAGCATCTTCGA-5′
1I	Arg146->Lys	Site-directed Priming	5′-GGGGCTACAAGGATAGATAT-3′
T1	COOH Truncation @ Trp179	Linker Insertion	5′-CGTGGTAATGATAGA-3′ 3′-CATTACTATCTTCGA-5′

*Designation

TABLE 2

ADP-RIBOSYLTRANSFERASE ACTIVITIES OF RECOMBINANT CTXA1 ANALOGS[1]

CTX MOLECULE	MUTATION	PROTEIN ADDED TO ASSAY(mG)[2]	SPECIFIC ACTIVTY FOR HEM G. PROTEIN[3]
Commercial CTXA1 (without urea)	none	1.00	1.00
Commercial CTXA1 (with urea)	none	1.11	0.53
rCTXA1/A7	none	1.56	0.56
rCTXA1/L7	Arg7->Lys	1.46	0
rCTXA1/1B	His44->Asn	1.47	0
rCTXA1/1C	His70->Asn	1.51	0.05
rCTXA1/1D	Glu112->Gln	1.65	0
rCTXA1/1E	Tyr6->Phe	1.04	1.01
rCTXA1/1F	Asp9->Glu	0.91	0.10
rCTXA1/1G	Pro185->Gln	1.23	0.81
rCTXA1/1H	Cys187->Ala	1.14	0.83
rCTXA1/1I	Arg146->Lys	1.05	0.83
rCTXA1/T1	Truncated at Trp179	1.85	<0.01

[1]The absolute amount of each protein used in each ADP-ribosyltransferase assay (se Figure 4) was estimated by densitometric scanning of the stained SDS-polyacrylamide g 1 (Figure 4, panel A) containing identical amounts of each protein used in the assay. The autoradiogram of the gel containing the human erythrocyte membranes (Figure 4, Panel C) was subsequently scanned to determine the radiographic density of the G protein alpha subunit ribosylated by each CTXA1 protein preparation. The density of the G protein band resulting from ADP-ribosylation with commercial CTXA1 without added urea was taken as 1.00 and the density of the band resulting form ribosylation by the other CTXA1 proteins was related to this preparation as a percentage of its density. These fractions were then normalized to 1.00 µg of added CTXA1 protein based on the densitometric of the stained gel to obtain an approximat , relative specific activity.

[2]Th amount of commercial CTXA1 (without added urea) in the assay was taken as 1.00 µg.

[3]Th radiographic density of the G protein alpha subunit ADP-ribosylated by the comm rcial CTXA1 (without added urea) was taken as 1.00.

Figure 4 shows a stained SDS-polyacrylamid gel (Panel A) of inclusion-body pr parations of rCTXA1 and its site-specific analogs. An amount of protein identical to that shown in this gel was used to catalyze the individual ADP-ribosyltransferase reactions. Trichloroacetic acid (TCA) precipitates from these reactions were also run in SDS-PAGE and the gels subjected to autoradiography to illuminate the [^{32}P]ADP-ribose-labeled substrates. Panel B illustrates the results of the reactions without added G protein-containing human erythrocyte membrane preparation and Panel C shows the reactions with this added substrate.

The most important finding of these experiments is found in Figure 4, Panel C (and confirmed in Panel B): certain site-specific amino acid residue substitutions result in diminishment and, in some cases, apparently complete loss of enzyme activity as measured in this assay. In this regard, rCTXA1/L7 (Arg^{9}→Lys), rCTXA1/1B (His^{44}→Asn) and rCTXA1/1D (Glu^{112}→Gln) analog subunits appear to possess virtually no enzyme activity, whereas analogs rCTXA1/1C (His^{70}→Asn) and rCTXA1/1F (Asp^{9}→Glu) appear to have reduced activity when compared with both native CTXA (with urea) and rCTX1/A7 (no mutation other than the methionine residue at the amino terminus). Truncation at Trp^{179} (rCTXA1/T1/1156) also results in an analog A subunit with severely diminished enzyme activity.

Although these autoradiographic assays of enzyme activity are not strictly quantitative, we have attempted to derive a quantitative assessment from the gel and autoradiograms of Figure 4 to illustrate in a numerical sense what can be visually observed. This evaluation is found in Table 2. Here, we subjected the stained SDS-polyacrylamide gel (Fig. 4, Panel A),

containing rCTXA1 and ach of the analogs described pr viously, to int grative scanning densitometry to more accurat ly ass ss the relativ amount of each protein added to the assay; these were related to the amount of A1 subunit in native CTXA (without urea) added to the assay, taken as a value of 1.00 µg. Although an attempt was made to add equivalent amounts of each protein to the assays (estimated on the basis of the percentage of subunit protein in each inclusion body preparation), it can be seen that this estimation may have lacked precision. The autoradiogram of the subsequent enzyme reactions with G protein substrate (Fig. 4, Panel C) was also subjected to densitometry to determine the relative density of the radiographic image of the radiolabeled G protein α subunit band with that labeled by native CTXA (no urea) taken as 100%. An approximate-relative specific activity was then calculated by dividing the image density by the amount of added enzyme, with the specific activity of native CTX (without urea) taken as 1.00. It should be noted that the results of this type of quantitation are subject to certain experimental limitations (e.g., assumption of equal dye staining by each of the subunit preparations, band selection and circumscription for digitized densitometry, densitometer response characteristics, and assumption of a linear relationship between [^{32}P]ADP-ribose labelling and radiographic density). Nevertheless, the results (Table 2) illustrate in a numerical manner what can be visually observed in the autoradiograms: marked diminishment of enzyme activity in analogs rCTXA1/1C (His^{70}→Asn), rCTXA1/1F (Asp^{9}→Glu), and rCTXA1/T1(Trp^{179} truncation) and virtual loss of activity by analogs rCTXA1/L7 (Arg^{9}→Lsy), rCTXA1/1B (His^{44}→Asn), and rCTXA1/1D (Glu^{112}→Gln).

In the case in which no exogenous substrate is added (Figur 4, Panel B), both nativ CTXA and the enzymatically- ctive CTXA1 prot ins can be seen to be autocatalytic, i.e., to catalyze the hydrolysis of NAD and the transfer of ADP-ribose to the enzyme itself (either in *cis*, in *trans*, or both). Multiple bands seen in the autoradiogram may be due to contaminating *E. coli* proteins capable of being ADP-ribosylated; alternatively, yet unlikely, they may represent minor variants of the subunit proteins (e.g., proteolytically-nicked or, perhaps, variants possessing some residual secondary structure in SDS). Recombinant CTXA1 preparations appear much more capable of participating in the autocatalytic process than does the A subunit of native CTX. The reasons for this increased autoribosylation are not presently understood, although it may be related to lack of substrate specificity by the yet-to-be-renatured recombinant protein, exposure of a sensitive ribosylation site in the recombinant protein as a result of improper secondary structure (no attempt was made in this particular experiment to achieve native conformation), or to the presence of ARFs (ADP-ribosylation factors) (31-37) in the crude recombinant preparations that stabilize the autocatalysis. However, when G protein substrate is added in the form of human erythrocyte membranes (Panel C), the focus of the ADP-ribosyltransferase reaction is shifted to this substrate, quenching autoribosylation.

Figure 5 demonstrates that the same general pattern of diminishment and loss of enzyme activity seen with the rCTXA1 analogs is also observed when the same residue substitutions are made in rCTXA versions of the r combinant subunit (i.e., versions with the A2 "tail" still coval ntly link d). However, the presence of the A2 region appears to significantly reduce the

ADP-ribosyltransferase of th enzymatically-active proteins. This r duction is mor clearly illustrated in Figure 6, in which identical amounts of rCTXA and rCTXA1 are evaluated in the enzyme assay (Panel A), the radiolabeled products run on the same gel, and consequently subjected to equivalent autoradiographic exposure times (Panel B). As can be seen, rCTXA1 appears to possess greater activity than rCTXA (compare lanes 7 and 4). Again, neither subunit construction with the $Arg^{9}\rightarrow Lys$ substitution (lanes 5 and 8) possess measurable ADP-ribosyltransferase activity for the G protein substrate. That this loss of enzyme activity in the analogs is not the result of *E. coli* contaminants suppressing catalysis is evident by the ability of native CTXA to ribosylate G protein in the presence of the *E. coli*-produced, analog-containing preparations (lanes 6 and 9).

Because of their reduction or essential elimination of a major marker of toxic activity (ADP-ribosyltransferase), the recombinant CTXA1 analog molecules produced by clones pCTXA1/L7/1156, pCTXA1/1B/1156, pCTXA1/1C/1156, pCTXA1/1D/1156, pCTXA1/1F/1156, and pCTXA1/T1/1156, as well as their rCTXA analog counterparts, are anticipated to have application alone or in combination with CTXB in safer vaccines. The described mutations would not be expected to reduce the normal, protective, immunogenic properties of native CTX subunits. The CTXA and CTXA1 analogs of this invention thus have application in combination with CTXB subunits in the form of a holotoxoid. The CTXB subunits may augment the immune response to CTXA and CTXA1, and vice-versa, and each may have protective epitopes. The CTXB subunits can be deriv d from *V. cholerae* or can be genetically-engine red subunits and their analogs. Gen tically-

engine r d subunit products can include fusion prot ins and non-fusion proteins.

Strategies identical to thos described above were employed to prepare additional recombinant analogs of the CTXA subunit of cholera toxin. The synthetic oligonucleotides utilized to effect codon substitutions, whether by linker mutagenesis or by mutagenesis by site-directed priming, are shown in Table 3. Briefly, analog CTXA1/1J ($Asp^{9}\rightarrow Tyr$) was prepared by linker mutagenesis as described for analog CTXA1/1F ($Asp^{9}\rightarrow Glu$), with the exception that the synthetic oligonucleotide possessed the appropriate codon substitution. For the construction of analogs CTXA1/1K ($Ser^{10}\rightarrow Gly$), CTXA1/1L ($Arg^{11}\rightarrow Lys$), and CTXA1/1M ($Arg^{11}\rightarrow His$), a novel DraII (also known as EcoO109I) restriction site was introduced into the CTXA1 gene by site-directed priming utilizing the following synthetic oligonucleotide primer:

5'-AGCAGTCAGGGGGCCTTATGCCAA-3'

Introduction of this site permitted linker mutagenesis in this region of the gene (using the previously-described NdeI site to the left of the insertion site and the newly-created DraII site to the right of the insertion site) to effect the codon changes that resulted in these three analogs. Site-directed priming was the method used to create the codon changes resulting in analogs CTXA1/1N ($His^{44}\rightarrow Tyr$), CTXA1/1"O" ($His^{44}\rightarrow Gln$), CTXA1/1P ($His^{44}\rightarrow Val$), CTXA1/1Q ($His^{70}\rightarrow Tyr$), CTXA1/1R ($His^{70}\rightarrow Gln$), and CTXA1/1S ($His^{70}\rightarrow Val$).

With two exceptions, each of these analogs was xpressed in recombinant *E. coli* and the isolated inclusion bodies were tested for their enzymatic ability to ADP-ribosylate either $G_s\alpha$ in human

rythrocyt membrane preparations or, specially in th cas of th His^{44} and His^{70} analogs, th ir ability to ADP-ribosylat $G_s\alpha$ and/or tubulin in membran preparations of H27 cultured human foreskin fibroblasts (provided by the University of California, San Francisco). The exceptions were for analog CTXA1/1J ($Asp^{9}\rightarrow Tyr$), which was recombinantly expressed but not assayed for activity, and analog CTXA1/1L ($Arg^{11}\rightarrow Lys$), for which a linker had been synthesized and cloning performed, but for which a correct-sequence clone had not been isolated.

The results of these analyses are presented in Figures 4 and 7, and are summarized in Table 4, Figure 4 provides comparative data for analogs reported in Table 1. Among the additional analogs described in Figure 7 and Table 4 are three having different substitutions at His^{44} (CTXA1/1N, CTXA1/1"O", CTXA1/1P). The absence of measurable enzyme activity in these analogs, in addition to lack of activity in previously-described analog CTXA1/1B ($His^{44}\rightarrow Asn$), indicates that these specific substitutions at His^{44} lead to inactivation of the subunit of cholera toxin possessing the intrinsic toxic activity of the multimeric molecule. Based on these results, it is likely that *any* substitution at this residue will produce such inactivation.

Three analogs (CTXA1/1Q, CTXA1/1R, CTXA1/1S) having substitutions for His^{70} are also among those described. These analogs are in addition to the analog CTXA1/1C ($His^{70}\rightarrow Asn$) of Table 1. As shown in Figure 7, all four His^{70} analogs possess *reduced* ability to ADP-ribosylate $G_s\alpha$ substrate, although they clearly *retain* the ability to ADP-ribosylate oth r non-$G_s\alpha$ prot in substrates (e.g., tubulin in H27 fibroblasts). Thus, substitutions for His^{70} result in apparent r duction of activity of CTXA1 for the

specific $G_s\alpha$ substrate believed to be involved in the pathognomonic cytotoxic response to cholera toxin. Such substitutions, if made in CTXA1 involved in a formed holotoxin multimer, would therefore likely result in an *attenuated* cholera toxin molecule as opposed to one totally lacking toxic properties.

Analysis of two additional analogs is shown in Figure 8. CTXA1/1K ($Ser^{10}\rightarrow Gly$) retains the catalytic activity associated with the native CTXA molecule. Substitution of His for Arg^{11} (CTXA1/1M) results in an analog having little or no measurable enzymatic activity. It would be expected that analog CTXA1/1L ($Arg^{11}\rightarrow Lys$) will also have significantly diminished activity when isolated and assayed, a conclusion which is supported by the findings of Table 1, (see $Arg^{7}\rightarrow Lys$).

TABLE 3
CONSTRUCTION OF CTXA1 ANALOGS

ANALOG	MUTATION	TECHNIQUE
CTXA1/1J	Asp9->Tyr	Linker Insertion
CTXA1/1K	Ser10->Gly	Linker Insertion
CTXA1/1L	Arg11->Lys	Linker Insertion
CTXA1/1M	Arg11->His	Linker Insertion
CTXA1/1N	His44->Tyr	Site-directed Priming
CTXA1/1"O"	His44->Gln	Site-directed Priming
CTXA1/1P	His44->Val	Site-directed Priming
CTXA1/1Q	His70->Tyr	Site-directed Priming
CTXA1/1R	His70->Gln	Site-directed Priming
CTXA1/1S	His70->Val	Site-directed Priming

ANALOG	OLIGONUCLEOTIDE SEQUENCE
CTXA1/1J	TAT GAA TGA TGA TAA GTT ATA TCG GGC ATA CT A CTT ACT ACT ATT CAA TAT AGC CCG TAT GAG ATC
CTXA1/1K	TAT GAA TGA TGA TAA GTT ATA TCG GGC AGA TGG CAG ACC TCC TGA TGA AAT AAA GCA GTC AGG G A CTT ACT ACT ATT CAA TAT AGC CCG TCT ACC GTC TGG AGG ACT ACT TTA TTT CGT CAG TCC CCC G
CTXA1/1L	TAT GAA TGA TGA TAA GTT ATA TCG GGC AGA TTC TAA GCC TCC TGA TGA AAT AAA GCA GTC AGG G A CTT ACT ACT ATT CAA TAT AGC CCG TCT AAG ATT CGG AGG ACT ACT TTA TTT CGT CAG TCC CCC G
CTXA1/1M	TAT GAA TGA TGA TAA GTT ATA TCG GGC AGA TTC TCA CCC TCC TGA TGA AAT AAA GCA GTC AGG G A CTT ACT ACT ATT CAA TAT AGC CCG TCT AAG AGT GGG AGG ACT ACT TTA TTT CGT CAG TCC CCC G
CTXA1/1N	CTT TAT GAT TAC GCA AGA GGA
CTXA1/1"O"	CTT TAT GAT CAG GCA AGA GGA
CTXA1/1P	CTT TAT GAT GTT GCA AGA GGA
CTXA1/1Q	AGA AGT GCC TAC TTA GTG GGT
CTXA1/1R	AGA AGT GCC CAG TTA GTG GGT
CTXA1/1S	AGA AGT GCC GTT TTA GTG GGT

TABLE 4

ADP-Ribosyltransferase Activities of CTXA1 Analogs

ANALOG	MUTATION	ADP-ribosyltransferase Activity [1]
CTXA1/1J	Asp9->Tyr	N.D.
CTXA1/1K	Ser10->Gly	[+]
CTXA1/1L	Arg11->Lys	N.D.
CTXA1/1M	Arg11->His	[-]
CTXA1/1N	His44->Tyr	[-]
CTXA1/1"O"	His44->Gln	[-]
CTXA1/1P	His44->Val	[-]
CTXA1/1Q	His70->Tyr	[±]
CTXA1/1R	His70->Gln	[±]
CTXA1/1S	His70->Val	[±]

[1] As visualized by SDS-PAGE and autoradiography: [+], full activity; [±], reduced activity; [-], no detectable activity; N.D., not determined.

IN VITRO ASSOCIATION OF rCTX SUBUNITS

A number of methods by which native cholera toxin can be dissociated and the individual subunits reassociated *in vitro* to reform the holotoxin molecules have been described in the literature(36,37). *In vitro* reassociation of the subunits of pertussis toxin has also been described in the literature for native subunits(38-40). Using a similar procedure, recombinant CTX subunits can be isolated, associated *in vitro* to form holotoxin-like species, and purified. In general, following expression and recovery, the individual subunits are combined in stoichiometric ratios (based on their relative content of specific subunit protein, if in the form of inclusion body preparations), approximating the ratio of subunits found in native CTX holotoxin. The preparation is solubilized in an aqueous solution containing a chaotropic agent or a detergent, or both. The preparation is subjected to reducing conditions (generally a reducing agent or a hydrogen atmosphere, or both) and then oxidized (with either an oxidizing agent or under an oxygen-enriched atmosphere, or both) to reform the necessary intramolecular disulfide bridges. Association of the subunits into holotoxin-like species is accomplished by diminishment or removal of the chaotropic or detergent solubilizing agent. This can be accomplished by a variety of means, to include filtration and buffer exchange by dialysis chromatography. The holotoxin-like species are then purified by conventional means, e.g., ion exchange, size-exclusion and affinity chromatography. It should be noted that B multimeric species, without the A subunit, may be recovered by similar means if inclusion-body preparations of the latter subunit are not added.

The genetically engineered analog subunits of this invention can be formulated, in a conventional manner, into a toxoided cholera vaccine. In the case of a toxin that has been "genetically" inactivated, such as cholera toxin in the present invention, further inactivating steps (such as chemical treatment or heat treatment) should not usually be required since these products are produced in non-pathogenic organisms and are inherently free of the enzyme activities that are generally accepted to elicit the adverse reactions to whole-cell cholera vaccines. Nevertheless, it is necessary to control purity of the recombinant product, particularly with regard to the endotoxin content. In general, recombinant holotoxoid, recombinant holotoxoid-like macromolecules, recombinant B subunit macromolecules, recombinant B subunit alone or possibly B subunit recombinant analogs, and even A subunit analogs alone described in the present invention as potential vaccinating antigens would be purified to ≥90% homogeneity. The nature and estimated quantity of contaminants, if any, would be evaluated to ensure that the extent of endotoxin contamination meets the standards of the individual regulatory agencies.

For purposes of parenteral delivery, the vaccine materials would normally be adsorbed onto aluminum adjuvants. This can be accomplished by at least two means: precipitation with preformed alum and precipitation with aluminum salts. The adsorbed precipitates are then resuspended in an excipient to yield a dosage concentration of vaccine antigen generally in the range of 5-100 μg per dose and an alum amount usually not exceeding 1.5 mg/dose; volume per dose is in the range of 0.1-1.0 ml. The suspending excipient is commonly a buffered solution (e.g., phosphate-buffered saline, pH 7.0), may have added stabilizers (e.g., glycerol), and will likely contain a

preservativ (e.g., 0.01% Thimerosol) to prevent microbial contamination and to extend shelf lif .

Th formulation and d liv ry of recombinant cholera toxoid, or subcomponents thereof, *via* live vector systems as also encompassed within this invention will depend upon the nature of that system. For example, oral delivery of recombinant (mutant) *V. cholerae*, *Salmonella sp.*, vaccinia virus, or adenovirus carrying genes for the A or A *and* B subunits, might well be encapsulated in enteric-coated delivery vehicles for passage to the gut or in aerosolizable forms (e.g., with liposomes) for targeting to the respiratory tract in order to elicit secretory immunoglobulin A antibodies for protection at mucosal surfaces. Alternatively, other oral forms of the vaccine can be prepared in accordance with procedures described in the literature, suitably adapted to accommodate the present antigenic agents. For instance, a recombinant *V. cholerae* strain can be lyophilized and mixed with a bicarbonate buffer to neutralize gastric acidity(41); or a holotoxoid in accordance with this invention can be used in the form of an effervescent tablet, appropriately buffered, to supplement a killed, whole-cell vaccine(1).

* * *

While this invention has been specifically illustrated in relation to recombinant production in *E. coli*, it will be appreciated by those skilled in the art that the principles for mutagenesis of the analog subunits as described herein may be employed in connection with other recombinant hosts and expression systems, and to produce other inactivated analogs of the toxin. Further, it should be understood that assembly of mutant analogs into a holotoxoid can take place in intact cells via homologous recombination, e.g., in *V. cholerae*, rather than *in vitro*. It is intended that the present invention include all modifications and improvements as come within the scope of the present invention as claimed.

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WHAT IS CLAIMED:

1. A recombinant DNA molecule, at least a portion of which encodes an analog of the catalytic subunit of cholera toxin, wherein said analog has reduced or no catalytic activity associated with cholera toxin reactogenicity.

2. The recombinant DNA molecule of claim 1, wherein the analog is of the A region of cholera toxin.

3. The recombinant DNA molecule of claim 1, wherein the analog is of the A1 subunit of cholera toxin.

4. The recombinant DNA molecule of claim 1, wherein the analog is capable of eliciting a cholera toxin-neutralizing immune response.

5. The recombinant DNA molecule of claim 1, which is obtained by site-specific mutagenesis resulting in an analog of the catalytic subunit which is less active or essentially inactive as determined by assay of ADP-ribosyltransferase activity.

6. The recombinant DNA molecule of claim 5, wherein the site-specific mutation is in the region bounded by the codons for methionine-1 and arginine-192 or serine-194, inclusively.

7. The recombinant DNA molecule of claim 6, which encodes an analog of the catalytic subunit comprising a site-specific mutation in one or more of th sit s of said subunit selected from among arginine-7, arginine-11, aspartic acid-9, histidin -44, histidine-70 and glutamic acid-112.

8. The recombinant DNA molecule of claim 6, which encodes an analog of the catalytic subunit comprising a truncation of the carboxyl-terminal portion beginning at tryptophan-179.

9. The recombinant DNA molecule of claim 1, which also encodes subunit B of cholera toxin.

10. A genetically engineered analog of the catalytic subunit of cholera toxin, said analog having reduced or essentially no catalytic activity associated with cholera toxin reactogenicity.

11. The analog of claim 10, which is of the A region of cholera toxin.

12. The analog of claim 10, which is of the A1 subunit of cholera toxin.

13. The analog of claim 10, which is capable of eliciting a cholera toxin-neutralizing immune response.

14. The analog of claim 10, which is obtained by site-specific mutagenesis resulting in a mutation of the catalytic subunit which is less active or essentially inactive as determined by assay of ADP-ribosyltransferase activity.

15. The analog of claim 14, wherein the site-specific mutation is in the region bounded by methionine-1 and arginine-192 or serine-194, inclusively.

16. The analog of claim 15, which comprises a site-specific mutation in one or more of the sites selected from among arginine-7, arginine-11, aspartic acid-9, histidine-44, histidine-70 and glutamic acid-112.

17. The analog of claim 15, which comprises a truncation of the carboxyl-terminal portion beginning at tryptophan-179.

18. An improved anti-cholera vaccine comprising an effective amount of an analog of the catalytic subunit of cholera toxin, wherein said toxin has a biological activity which (a) can elicit a cholera toxin-neutralizing immune response and (b) has reduced or essentially no catalytic activity associated with cholera toxin reactogenicity.

19. The improved vaccine of claim 18, wherein the analog is of the A region of cholera toxin.

20. The improved vaccine of claim 18, wherein the analog is of the A1 subunit of cholera toxin.

21. The improved vaccine of claim 18, wherein the toxin-neutralizing immune response provides immunoprotection against cholera disease.

22. The improved vaccine of claim 18, wherein the analog has been derived by site-specific mutagenesis resulting in a mutation of the catalytic subunit of cholera toxin which has less or essentially no ADP-ribosyltransferase activity.

23. The improved vaccine of claim 22, wherein the site-specific mutation is in the region of the catalytic subunit bounded by methionine-1 and arginine-192 or serine-194, inclusively.

24. The improved vaccine of claim 23, wherein the site-specific mutation is in one or more of the sites of said subunit selected from among arginine-7, arginine-11, aspartic acid-9, histidine-44, histidine-70, and glutamic acid-112.

25. The improved vaccine of claim 23, wherein the mutation comprises truncation of the carboxyl-terminal portion beginning at tryptophan-179.

26. The improved vaccine of claim 18, wherein the analog of the catalytic subunit is associated with the B oligomer.

27. The improved vaccine of claim 26, wherein the B oligomer is the native form.

28. The improved vaccine of claim 26, wherein the B oligomer has been genetically engineered.

29. A prokaryotic or eukaryotic cell transformed with a DNA molecule according to claim 1 which is capable of expressing the polypeptide product or products encoded by said DNA molecule.

30. An *E. coli* host cell according to claim 29.

31. A strain of *Vibrio cholerae* containing a genetically engineered toxin operon in which one or more specific amino acid residues responsible for cholera toxin reactogenicity have been altered or deleted by mutagenesis.

32. The strain of *Vibrio cholerae* according to claim 31, wherein the operon has been mutagenized to alter one or more amino acids selected from among arginine-7, arginine-11, aspartic acid-9, histidine-44, histidine-70, and glutamic acid-112, or to truncate the carboxyl terminal portion beginning at tryptophan-179.

33. A method for the production of an analog of the catalytic subunit of cholera toxin which has reduced or essentially no catalytic activity associated with cholera toxin reactogenicity, comprising:

(a) identifying one or more amino acid residues of the toxin which are associated with such catalytic activity;

(b) effecting site-directed mutagenesis of the toxin cistron or operon to remove or replace such residue or residues and produce a mutagenized cistron or operon, and

(c) expressing the mutagenized cistron or operon in a transformed organism to produce a toxoid characterized by reduced or essentially no catalytic activity.

34. An isolated, genetically engineered protein having substantially the amino acid sequence of Figure 1A starting with asparagine-1 of the mature sequence and ending with leucine-240.

35. The protein of claim 34 which has been derived by expression in a host other than *Vibrio cholera*.

36. The protein of claim 35 which has been expressed in *E. coli*.

S
s
p
I
N
d
e
I
preA
mature A
1 ATGGTAAAGATAATATTTGTGTTTTTTATTTTCTTATCATCATTTTCATATGCAAATGAT
TACCATTTCTATTATAAACACAAAAAATAAAAGAATAGTAGTAAAAGTATACGTTTACTA 60
M V K I I F V F F I F L S S S F S Y Y A N N D -
X
b
a
I
61 GATAAGTTATATCGGGCAGATTCTAGACCTCCTGATGAAATAAAGCAGTCAGGTGGTCTT 120
CTATTCAATATAGCCCGTCTAAGATCTGGAGGACTACTTTATTTCGTCAGTCCACCAGAA
D K L Y R A D S R P P D E I K Q S G G L -
T
a
S B
c q c
a I 1
I I I
121 ATGCCAAGAGGACAGAGTGAGTACTTTGACCGAGGTACTCAAATGAATATCAACCTTTAT 180
TACGGTTCTCCTGTCTCACTCATGAAACTGGCTCCATGAGTTTACTTATAGTTGGAAATA
M P R G Q S E Y F D R G T Q M N I N L Y -
181 GATCATGCAAGAGGAACTCAGACGGGATTTGTTAGGCACGATGATATGTTTCCACC 240
CTAGTACGTTCTCCTTGAGTCTGCCCTAAACAATCCGTGCTACTACCTATACAAAGGTGG
D H A R G T Q T G F V R H D D G Y V S T -

FIG. 1A

```
                    B
                    s     D
                    p     rP
                    1     af
                    2     Il
                    8     IM
                    6     II
                    I      /
241 TCAATTAGTTTGAGAAGTGCCCACTTAGTGGGTCAAACTATATTGTCTGGTCATTCTACT
    AGTTAATCAAACTCTTCACGGGTGAATCACCCAGTTTGATATAACAGACCAGTAAGATGA 300
    S  I  S  L  R  S  A  H  L  V  G  Q  T  I  L  S  G  H  S  T  -

                                A
                                f     N     T
                                l     s     a
                                I     p     q
                                I     H     I
                                I     I     I
301 TATTATATATATGTTATAGCCACTGCACCCAACATGTTTAACGTTAATGATGTATTAGGG
    ATAATATATATACAATATCGGTGACGTGGGTTGTACAAATTGCAATTACTACATAATCCC 360
    Y  Y  I  Y  V  I  A  T  A  P  N  M  F  N  V  N  D  V  L  G  -

361 GCATACAGTCCTCATCCAGATGAACAAGAAGTTTCTGCTTTAGGTGGGATTCCATACTCC
    CGTATGTCAGGAGTAGGTCTACTTGTTCTTCAAAGACGAAATCCACCCTAAGGTATGAGG 420
    A  Y  S  P  H  P  D  E  Q  E  V  S  A  L  G  G  I  P  Y  S  -
```

FIG. 1A CONT.'

```
      BstXI               Tth111II
421 CAAATATATGGATGGTATCGAGTTCATTTTGGGGTGCTTGATGAACAATTACATCGTAAT 480
    GTTTATATACCTACCATAGCTCAAGTAAAACCCCACGAACTACTTGTTAATGTAGCATTA
    Q  I  Y  G  W  Y  R  V  H  F  G  V  L  D  E  Q  L  H  R  N  -

                              GsuI                       BspMI
481 AGGGGCTACAGAGATAGATATTACAGTAACTTAGATATTGCTCCAGCAGCAGATGGTTAT 540
    TCCCCGATGTCTCTATCTATAATGTCATTGAATCTATAACGAGGTCGTCGTCTACCAATA
    R  G  Y  R  D  R  Y  Y  S  N  L  D  I  A  P  A  A  D  G  Y  -

              BspMII         Ksp632I     DsaI
541 GGATTGGCAGGTTTCCCTCCGGAGCATAGAGCTTGGAGGGAAGAGCCGTGGATTCATCAT 600
    CCTAACCGTCCAAAGGGAGGCCTCGTATCTCGAACCTCCCTTCTCGGCACCTAAGTAGTA
    G  L  A  G  F  P  P  E  H  R  A  W  R  E  E  P  W  I  H  H  -
```

FIG. 1A CONT.'

second proposed
B authentic
s COOH
m end
I A1
C
1
a
I
start
A2
first proposed
authentic COOH
end and recombinant
end of A1
S
s
p
I
End A2
601
660
661
720
721
777
GCACCGCCCGGGTTGTGGGAATGCTCCAAGATCATCGATGAGTAATACTTGCGATGAAAAA
CGTGGCGGGCCCAACACCCTTACGAGGTTCTAGTAGCTACTCATTATGAACGCTACTTTTT
A P P G C G N A P R S S S M S N T C D E K -
T Q S L G V K K F L L D E Y Q Q S K K V K R R Q I -
ACCCAAAGTCTAGGTGTAAAATTCCTTGACGAATACCAATCTAAAGTTAAAAGACAAATA
TGGGTTTCAGATCCACATTTTAAGGAACTGCTTATGGTTAGATTTCAATTTTCTGTTTAT
T Q S L G V K K F L L D E Y Q Q S K K V K R R Q I -
TTTTCAGGCTATCAATCTGATATTGATACACATAATAGAATTAAGGATGAATTATGA
AAAAGTCCGATAGTTAGACTATAACTATGTGTATTATCTTAATTCCTACTTAATACT
F S G Y Q S D I D T H N R I K D E L * -

FIG. 1A CONT.'

E
c
o
5
7
I
PreB
1
ATGATTAAATTAAAATTTGGTGTTTTTTTTTACAGTTTTACTATCTTCAGCATATGCACAT
TACTAATTTAATTTTTAAACCACACAAAAAAATGTCAAAATGATAGAAGTCGTATACGTGTA 60
M I K L K F G V V F F T T V L L L S S A Y Y A T -
S
s
p
I
Mature
B
GGAACACCTCAAAATATTACTGATTTGTGTGCAGAATACCACACAACACAAATATATACG 120
CCTTGTGGAGTTTTATAATGACTTAAACACACGTCTTATGGTGTTGTGTTTATATATGC
G T P Q N I T D L C A E Y H N T Q I Y T -
AX
cc
ca
II
CTAAATGATAAGATATTTTCGTATACAGAATCTCTAGCTGGAAAAAGAGATGGCTATC 180
GATTTACTATTCTATAAAAGCATATGTCTTAGAGATCGACCTTTTTCTCTACCGATAG
L N D K I F S Y T E S L A G K R E M A I -

FIG. 1B

```
                                                                  H
                                                                  i
                                                                  n
                                                                  c
                                                                  I
                                                                  I
181 ATTACTTTTAAGAATGGTGCAATTTTTCAAGTAGAAGTACCAAGTAGTCAACATATAGAT 240
    TAATGAAAATTCTTACCACGTTAAAAAGTTCATCTTCATGGTTCATCAGTTGTATATCTA
     I  T  F  K  N  G  A  I  F  Q  V  E  V  P  S  S  S  Q  H  I  D  .  -

                                                        A
                                                        o
                                                        c
                                                        I
241 TCACAAAAAAAAGCGATTGAAAGGATGAAGGATACCCTGAGGATTGCATATCTTACTGAA 300
    AGTGTTTTTTTTCGCTAACTTTCCTACTTCCTATGGGACTCCTAACGTATAGAATGACTT
     S  Q  K  K  A  I  E  R  M  K  D  T  L  R  I  A  Y  L  T  E  -

                                                  E
                                                  c
                                                  o
                                                  5
                                                  7
                                                  I
301 GCTAAAGTCGAAAAGTTATGTGTATGGAATAATAAAACGCCTCATGCGATTGCCGCAATT 360
    CGATTTCAGCTTTTCAATACACATACCTTATTATTTTGCGGAGTACGCTAACGGCGTTAA
     A  K  V  E  K  L  C  V  W  N  N  K  T  P  H  A  I  A  A  I  -

        End B
361 AGTATGGCAAATTAA 375
    TCATACCGTTTAATT
     S  M  A  N  *  -
```

FIG. 1B CONT.'

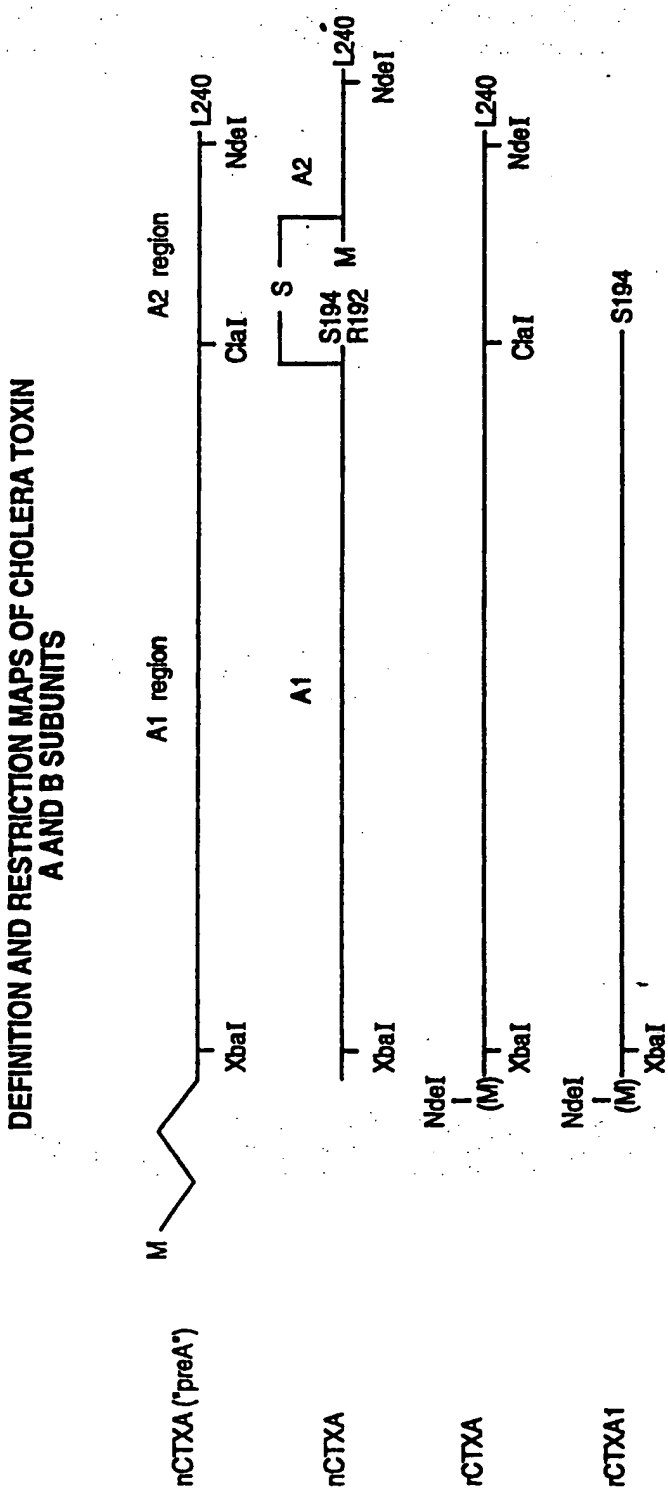

DEFINITION AND RESTRICTION MAPS OF CHOLERA TOXIN A AND B SUBUNITS
A1 region
A2 region
M
XbaI
ClaI
L240
NdeI
S
S194
R192
A2
A1
nCTXA ("preA")
nCTXA
rCTXA
rCTXA1
nCTXB ("preB")
nCTXB
rCTXB
(M)
AccI
N103

FIG. 2

FIG. 3

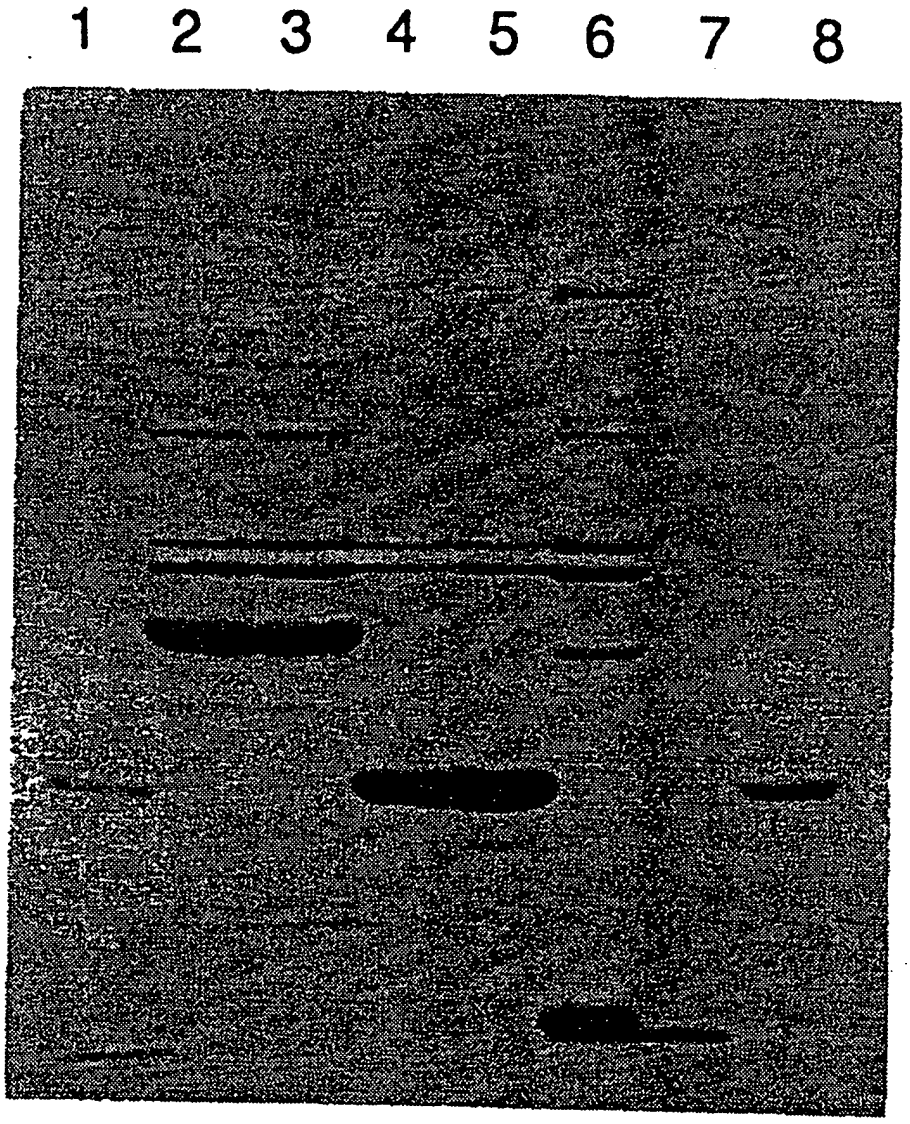


FIG. 4A

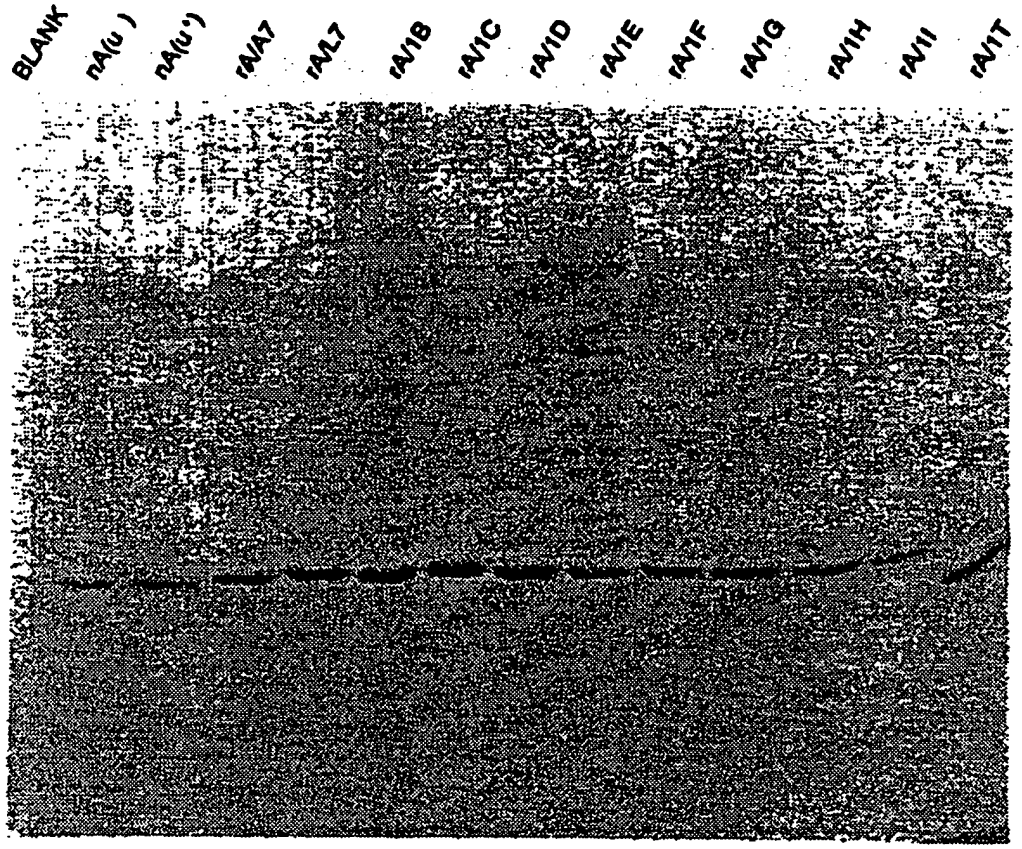

BLANK
rA(u)
rA(u')
rA/A7
rA/L7
rA/1B
rA/1C
rA/1D
rA/1E
rA/1F
rA/1G
rA/1H
rA/1I
rA/1T

FIG. 4B

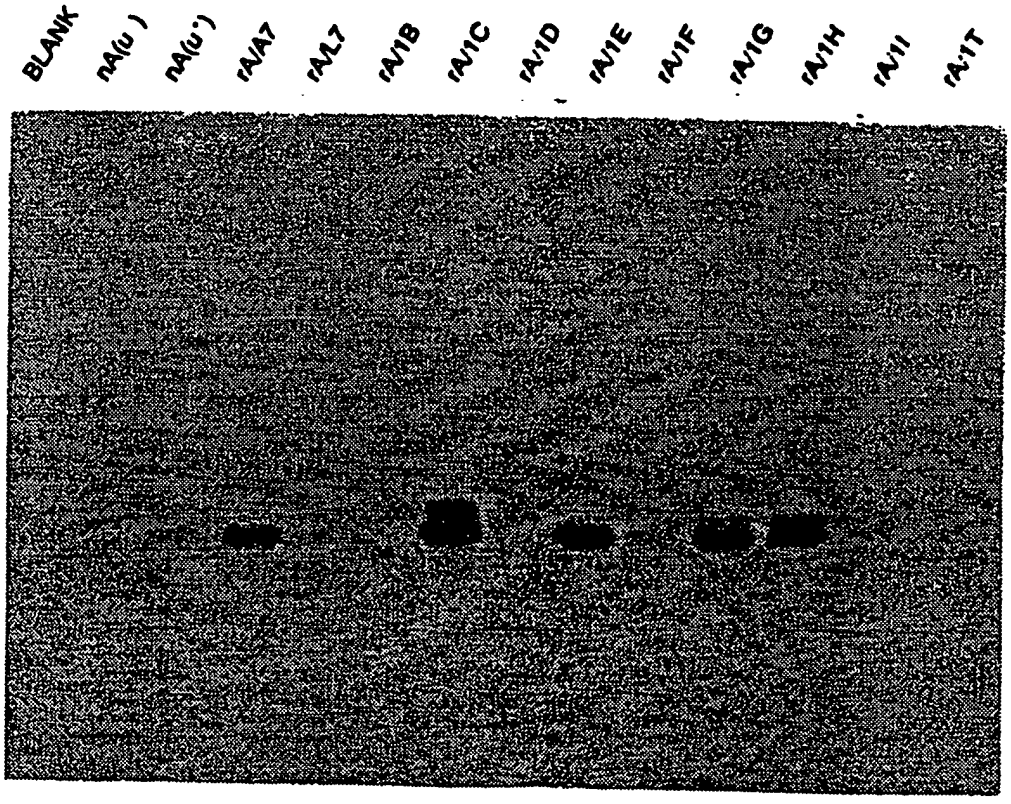

BLANK
rA(u)
rA(u')
rA/A7
rA/L7
rA/1B
rA/1C
rA/1D
rA/1E
rA/1F
rA/1G
rA/1H
rA/1I
rA/1T

FIG. 4C

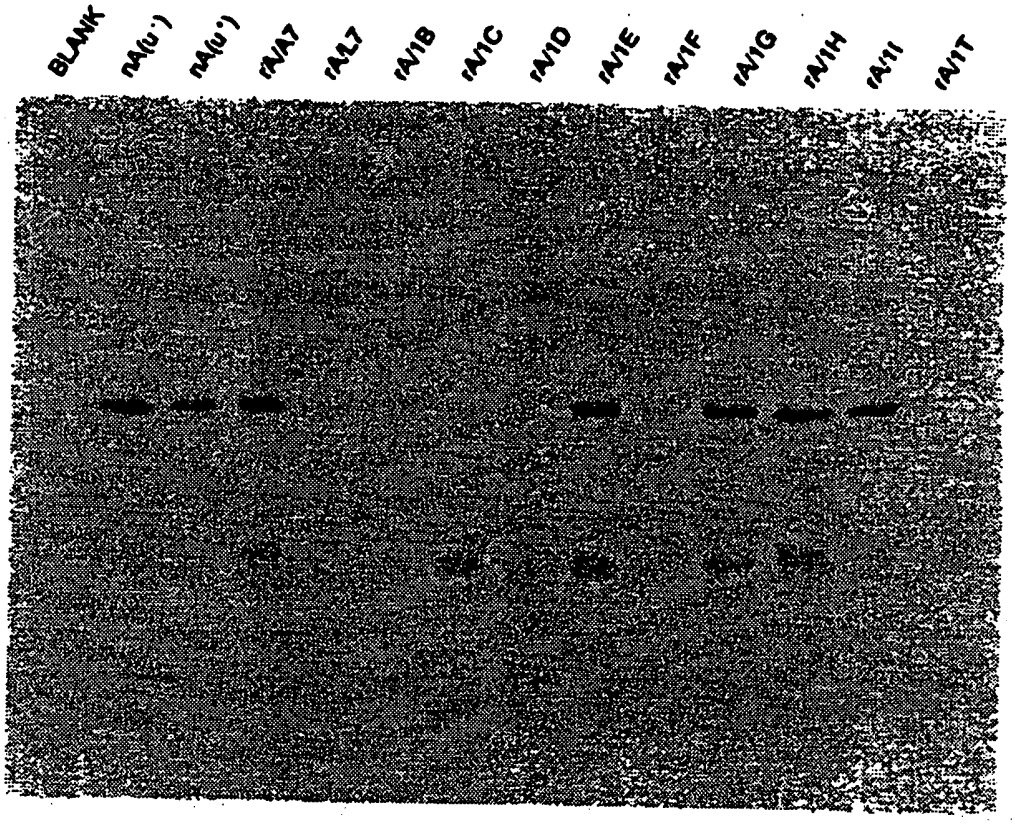

BLANK
nA(u⁻)
nA(u⁺)
rA/A7
rA/L7
rA/1B
rA/1C
rA/1D
rA/1E
rA/1F
rA/1G
rA/1H
rA/1I
rA/1T

FIG. 5A

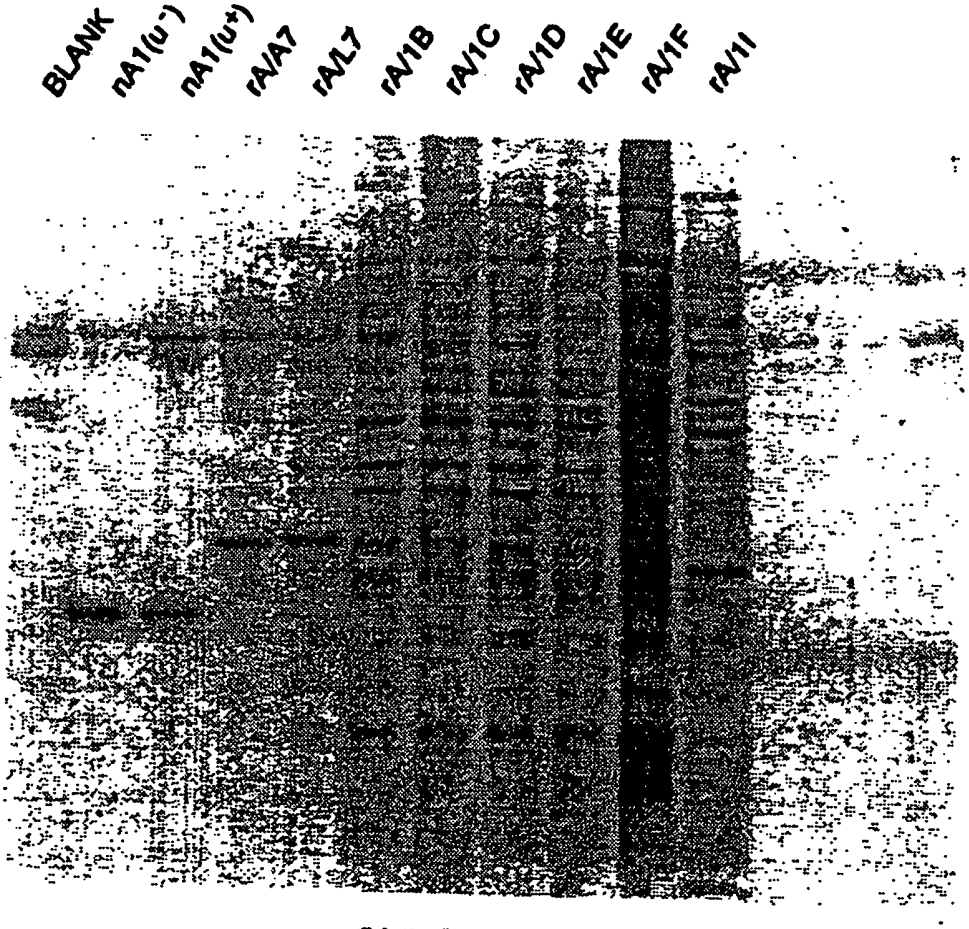

BLANK
nA1(u⁻)
nA1(u⁺)
rA/A7
rA/L7
rA/1B
rA/1C
rA/1D
rA/1E
rA/1F
rA/1I

FIG. 5B

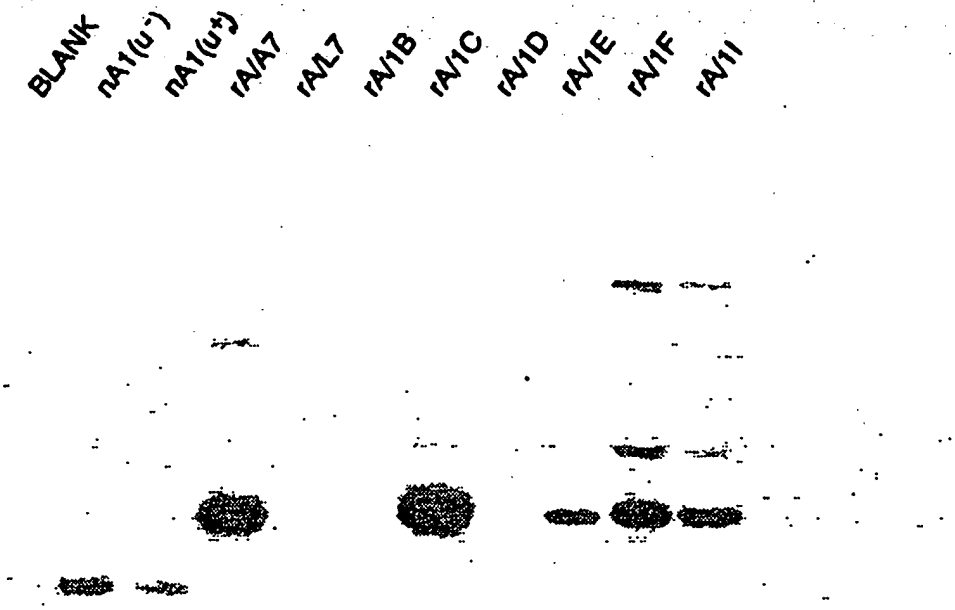

BLANK
nA1(u⁻)
nA1(u⁺)
rA/A7
rA/L7
rA/1B
rA/1C
rA/1D
rA/1E
rA/1F
rA/1I

FIG. 5C

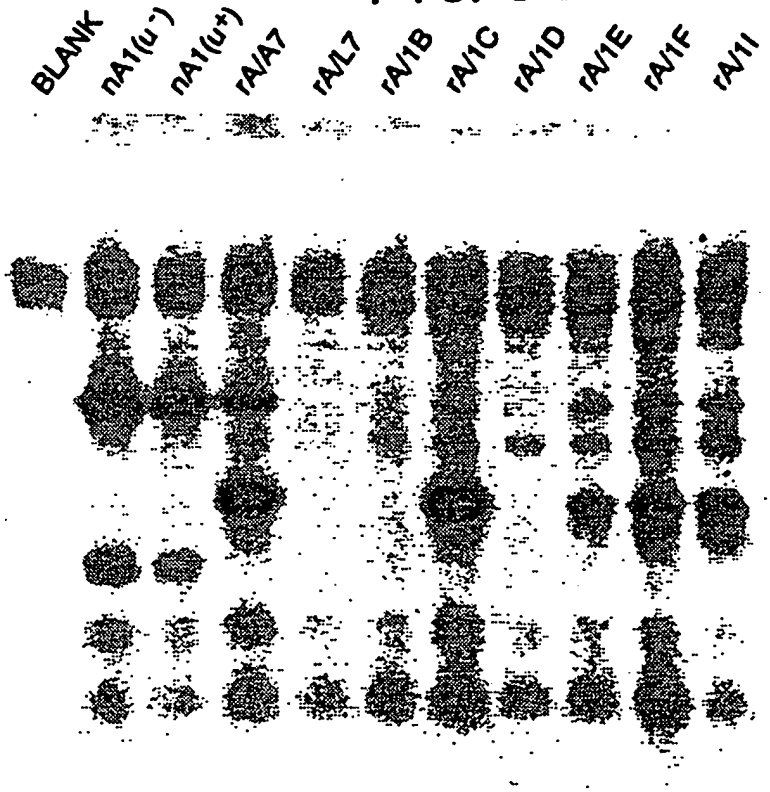

BLANK
nA1(u⁻)
nA1(u⁺)
rA/A7
rA/L7
rA/1B
rA/1C
rA/1D
rA/1E
rA/1F
rA/1I

FIG. 6A

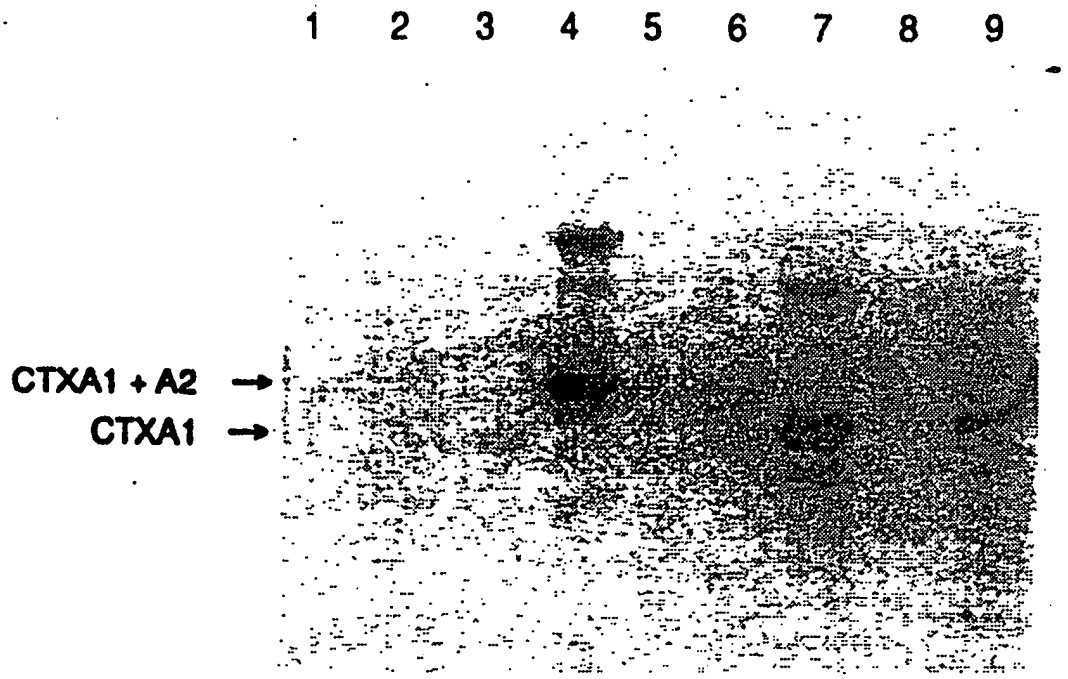
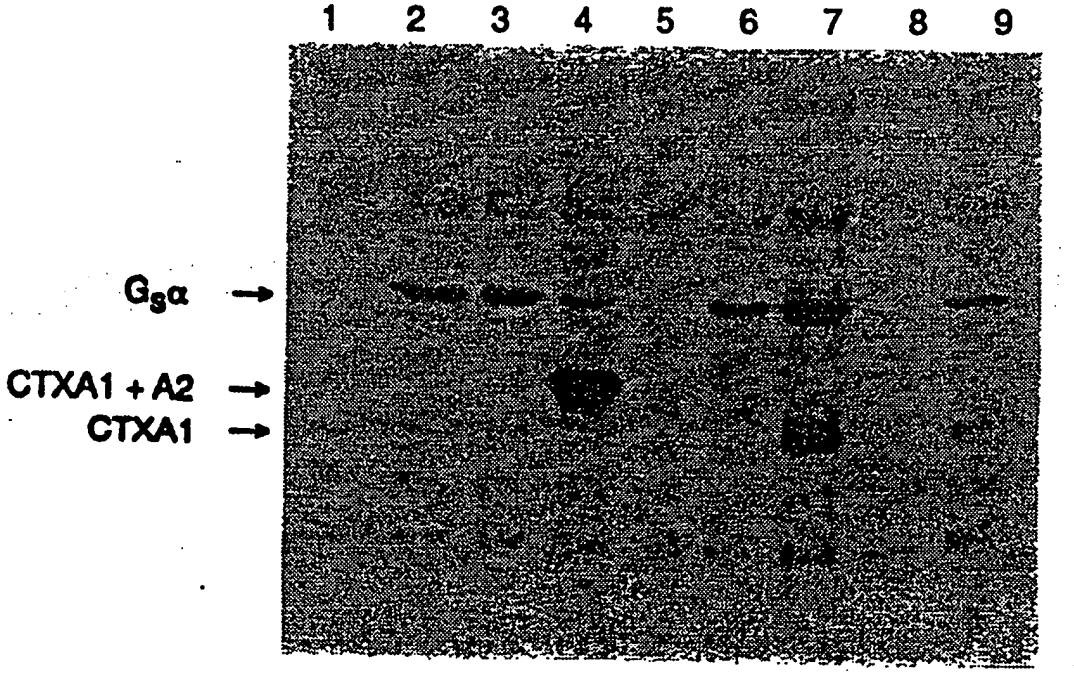


FIG. 6B



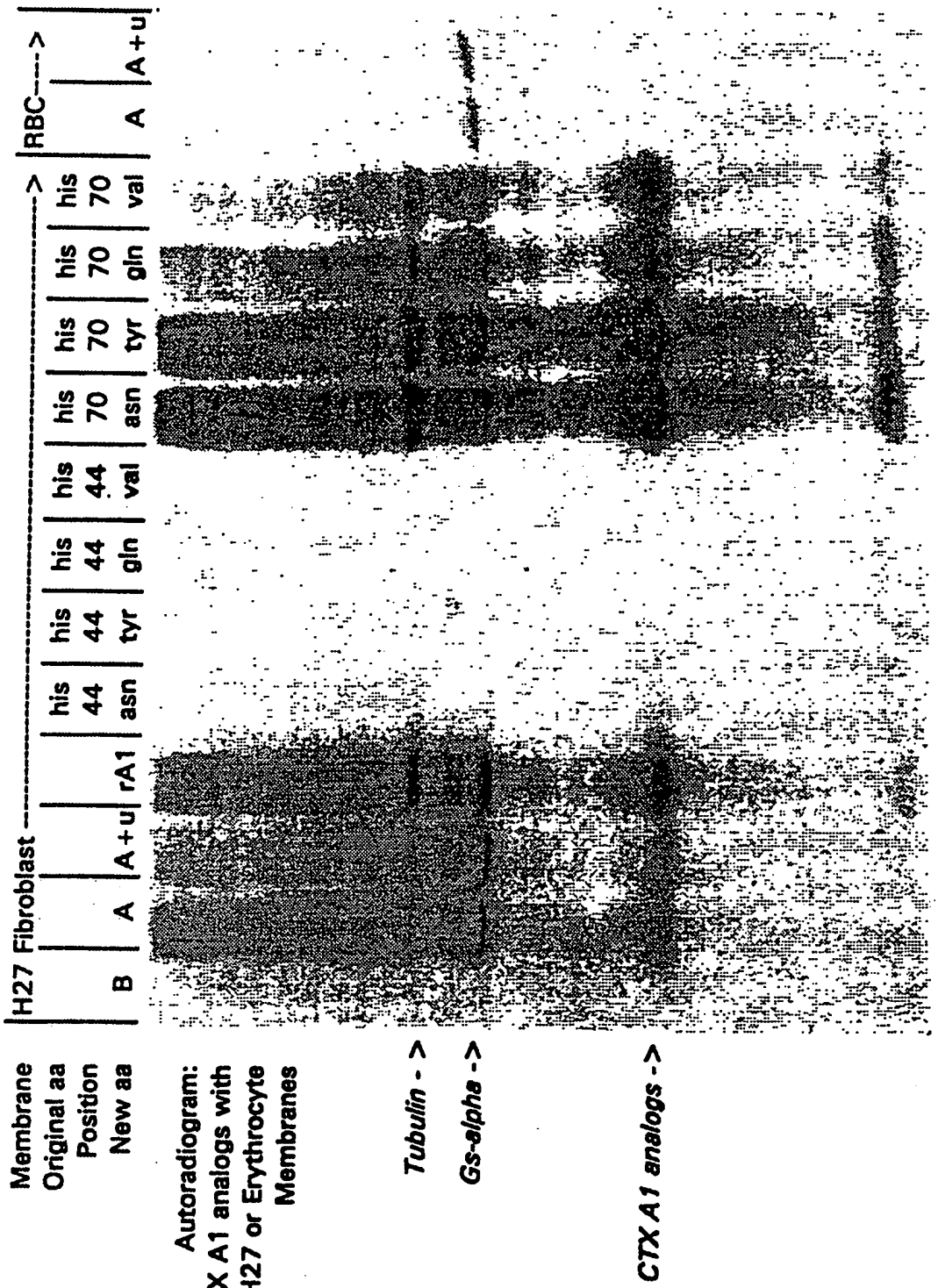
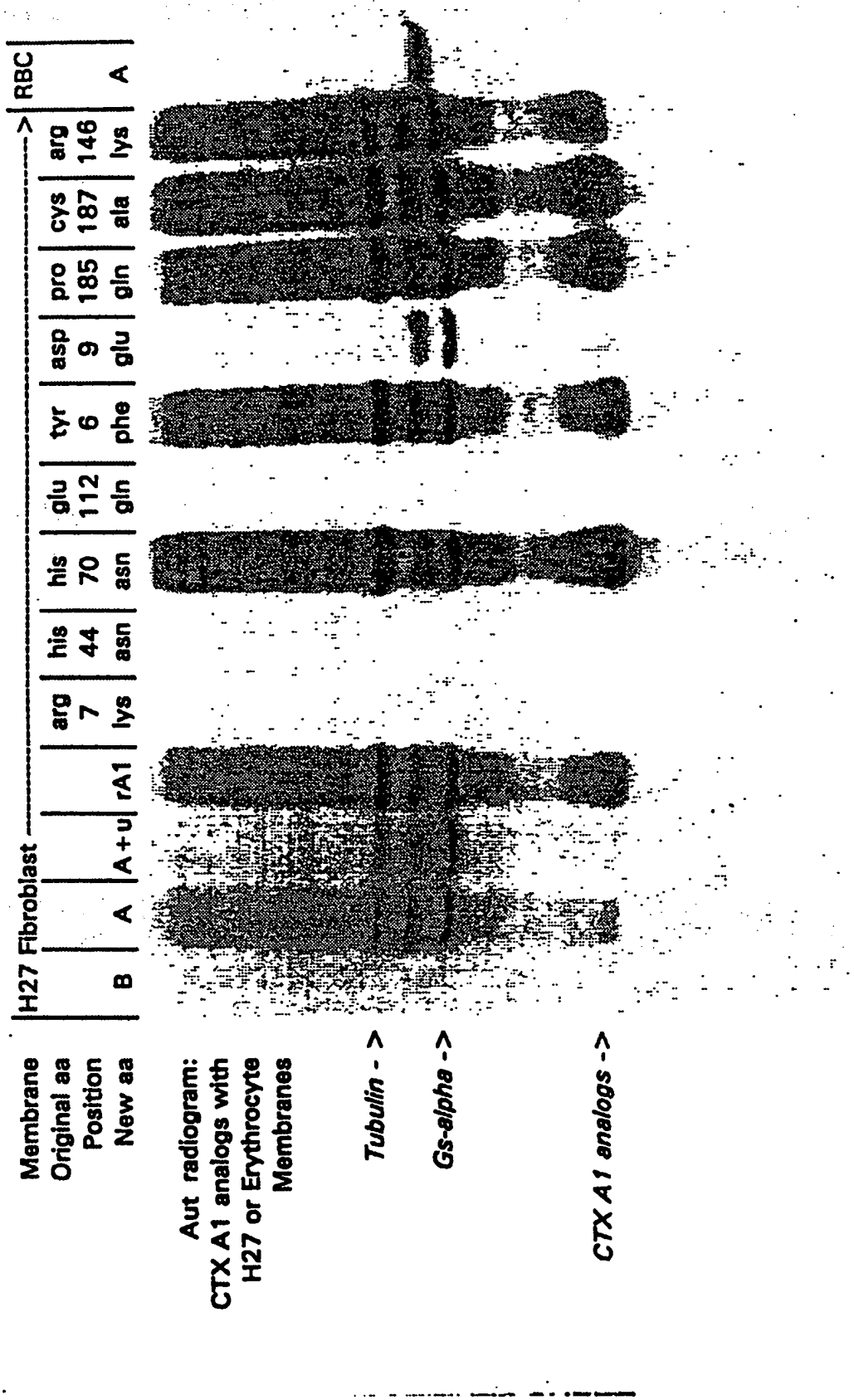

Membrane
Original aa
Position
New aa
H27 Fibroblast
B
A
A+u
rA1
his 44 asn
his 44 tyr
his 44 gln
his 44 val
his 70 asn
his 70 tyr
his 70 gln
his 70 val
RBC
A
A+u
Autoradiogram:
CTX A1 analogs with
H27 or Erythrocyte
Membranes
Tubulin ->
Gs-alpha ->
CTX A1 analogs ->
Substitute

FIG. 7A


Membrane
Original aa
Position
New aa
H27 Fibroblast
B
A
A+u
rA1
arg 7 lys
his 44 asn
his 70 asn
glu 112 gln
tyr 6 phe
asp 9 glu
pro 185 gln
cys 187 ala
arg 146 lys
RBC
A
Aut radiogram:
CTX A1 analogs with
H27 or Erythrocyte
Membranes
Tubulin ->
Gs-alpha ->
CTX A1 analogs ->
FIG. 7B

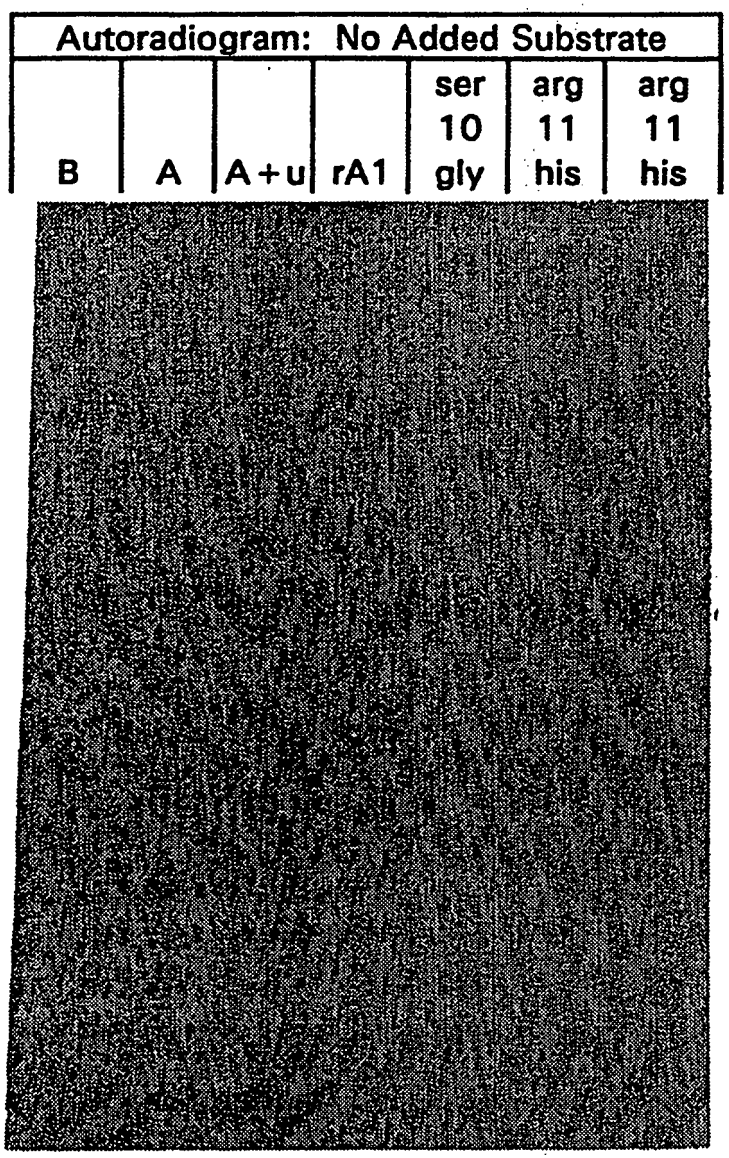

Autoradiogram: No Added Substrate
B
A
A+u
rA1
ser 10 gly
arg 11 his
arg 11 his

FIG. 8B

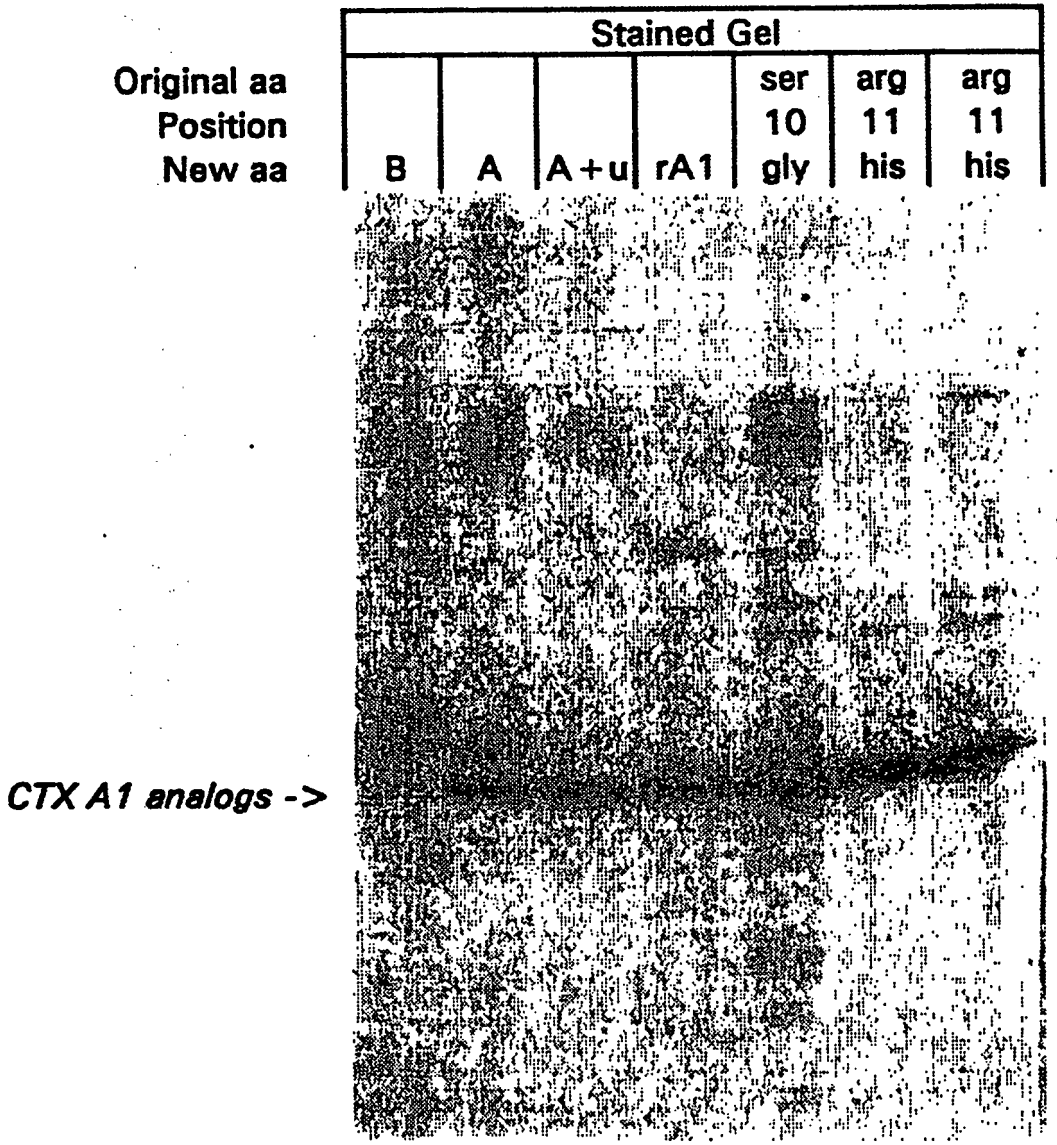

Stained Gel
Original aa
Position
New aa
B
A
A+u
rA1
ser 10 gly
arg 11 his
arg 11 his
CTX A1 analogs ->>

FIG. 8A

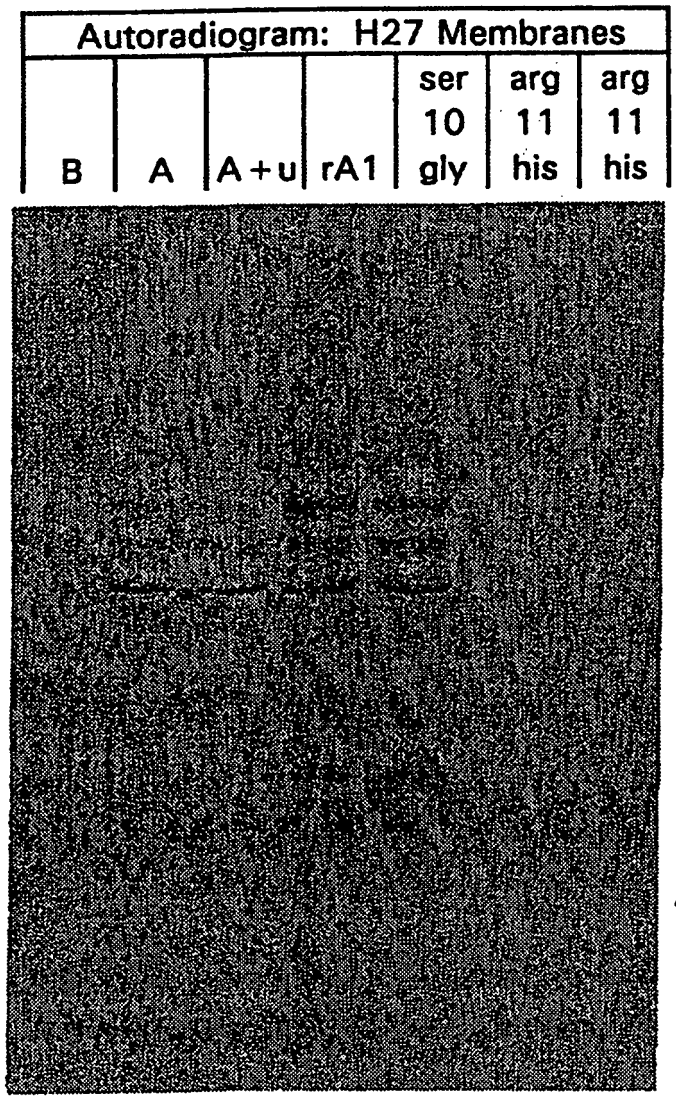

Autoradiogram: H27 Membranes
B
A
A+u
rA1
ser 10 gly
arg 11 his
arg 11 his

FIG. 8D

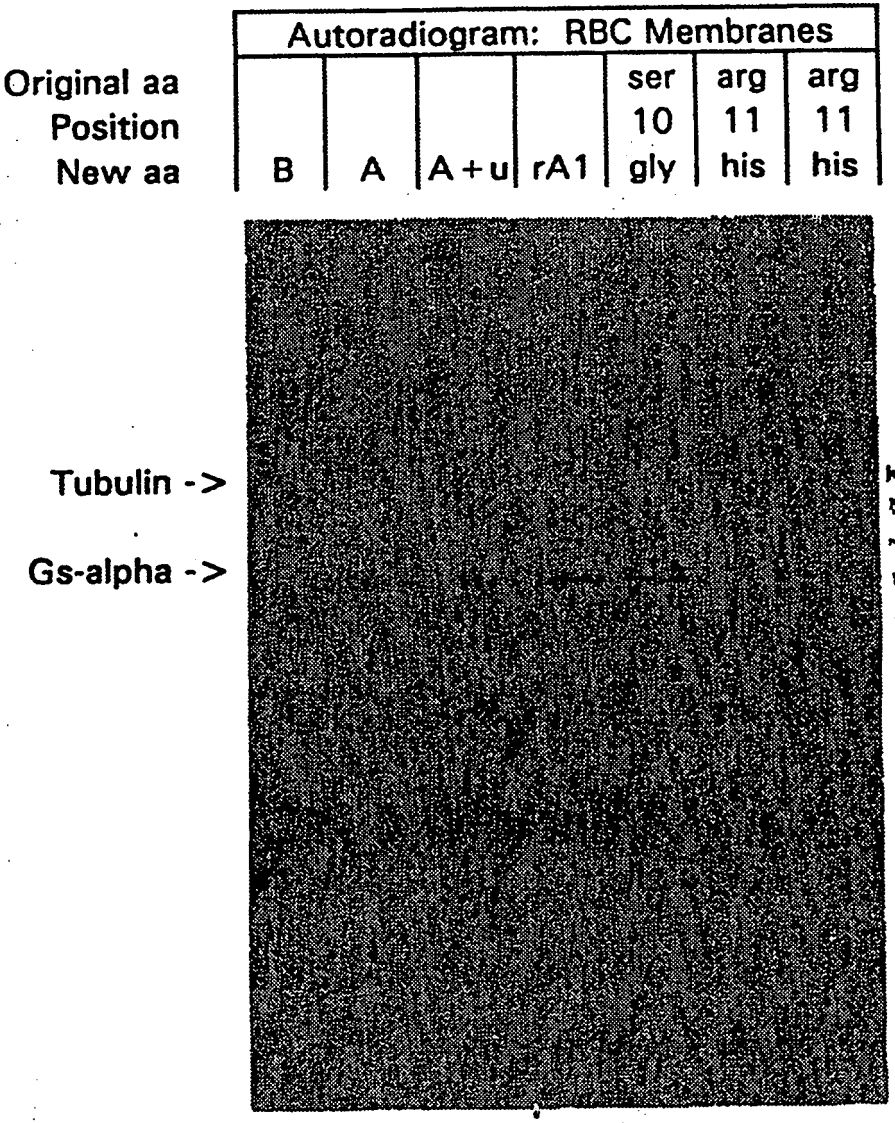

Autoradiogram: RBC Membranes
Original aa
Position
New aa
B
A
A+u
rA1
ser 10 gly
arg 11 his
arg 11 his
Tubulin ->
Gs-alpha ->
16/116

FIG. 8C

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/106; C12N 1/20, 1/21, 5/10, 9/12, 15/31, 15/09; C12P 21/02

US CL :435/172.3, 194, 240.2, 252.1, 252.3, 252.33; 424/88; 536/27

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 194, 240.2, 252.1, 252.3, 252.33, 909, 69.3, 71.1, 172.1; 424/88; 536/27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE, BIOSIS
search terms: cholera toxin, reactogenicity, mutant, analog, toxoid

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim N .
X	RESEARCH IN MICROBIOLOGY, Volume 141, issued 1990, J.B. Kaper et al., "Recombinant Attenuated Vibrio cholerae Strains Used as Live Oral Vaccines". pages 901-906, entire document especially pages 903-904.	1-6, 9-11, 13-15, 29, 31, 33
P,Y	CHEMICAL ABSTRACTS, Volume 115, No. 17, issued 28 October 1991, Y. Lobet et al., "Effect of Site-directed Mutagenic Alterations on ADP-Ribosyltransferase Activity of the A-subunit of Escherichia coli Heat-labile Enterotoxin", pages 433-434, abstract no. 178165, Infection and Immunity, 59(9), pages 2870-2879.	1-7, 9-16, 18-24, 26-33
X A	US, A, 4,935,364 (Kaper et al.) 19 June 1990, entire document.	33 1-32
X A	US, A, 4,882,278 (Mekalanos) 21 November 1989, entire document.	33 1-32
X Y	US, A, 4,666,837 (Harford et al.) 19 May 1987, entire document.	34-36 1-33
A	US, A, 4,328,209 (Finkelstein et al.) 04 May 1982.	18-28

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 13 August 1992	Date of mailing of the international search report 19 AUG 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer REBECCA PROUTY Telephone N . (703) 303-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X Y	CHEMICAL ABSTRACTS, Volume 116, N . 7, issued 17 February 1992, W. Burnette et al., "Site-specific Mutagenesis of the Catalytic Subunit of Cholera Toxin: Substituting Lysine for Arginine 7 Causes Loss of Activity", page 273, abstract no. 53261, Infection and Immunity, 59(11), pages 4266-4270.	1-7, 10-16, 29-30, 33-36 8, 17-29, 31-32
Y	SCIENCE, Volume 232, issued 06 June 1986, C. Locht et al., "Pertussis Toxin Gene: Nucleotide Sequence and Genetic Organization", pages 1258-1264, especially page 1261-1262 paragraphs 1 and 6 and Fig. 3	1-33
Y	SCIENCE, Volume 242, issued 07 October 1988, W.N. Burnette et al., "Pertussis Toxin S1 Mutant With Reduced Enzyme Activity and a Conserved Protective Epitope", pages 72-74, entire document.	1-33